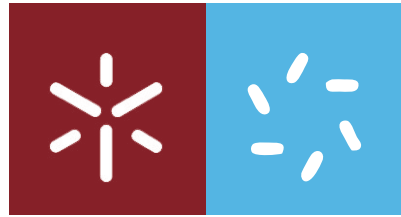


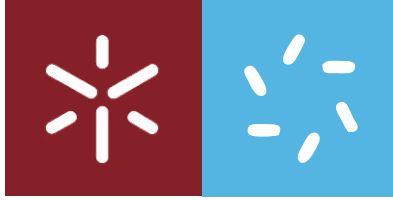


Ana Rita Pimentão Rodrigues

**The role of specialists vs. generalists in
ecosystem processes under environmental
stress**

Universidade do Minho
Escola de Ciências





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ecosystem processes under environmental
stress**

Master thesis in Ecology

Work made under the orientation of

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Abstract

In low order forested streams, where insufficient sunlight penetrates through the canopies, plant detritus from the riparian vegetation constitutes the major source of energy for freshwater organisms. The decomposition of this material is a key process in freshwater ecosystems and is mainly driven by aquatic microbial decomposers and invertebrate detritivores. Fungi, particularly aquatic hyphomycetes, seem to have a major role in organic matter turnover by constituting a link between plant litter and invertebrate detritivores in detritus-based food webs. Human activities are threatening biodiversity and altering ecosystems at a worldwide scale. The widespread and extensive use of antifungal formulations, which include agrochemicals and pharmaceuticals, may affect non-target microbial decomposer communities. We evaluated the effects of tebuconazole, clotrimazole and terbinafine on freshwater ecosystems using leaf litter decomposition and a decomposer-shredder-collector system model.

In a first experiment, the direct effects of the toxicants were evaluated on fungal biomass and sporulation, as well as on leaf litter decomposition. Alder leaves (12 mm diameter disks) were colonized by fungi in an unpolluted stream for 14 days, and then exposed in laboratory microcosms to 8 concentrations of each fungicide (from 10 to 1280 $\mu\text{g L}^{-1}$). Under control conditions, 8 sporulating fungal species were identified, with *Articulospora tetracladia* and *Flagellospora curvula* being dominant. Fungicide exposure led to an overall reduction in the diversity and richness of fungal community, and to shifts in species dominance. Tebuconazole and clotrimazole significantly reduced fungal biomass and reproduction, while terbinafine stimulated fungal sporulation and had no effects on fungal biomass. Moreover, none of the tested fungicides affected leaf decomposition rates.

In a second experiment, the indirect effects of the fungicides were assessed on the next trophic level (detritivore invertebrates), by evaluating leaf mass loss promoted by a specialist (shredder, *Allogamus* sp.) and a generalist (collector, *Chironomus riparius*). Leaf discs from the previous experiment were given to invertebrates to assess if leaves pre-exposed to the fungicides affected the feeding behaviour of invertebrates. Overall, the feeding activity of *C. riparius* and *Allogamus* sp. was not affected by exposure to fungicide-contaminated leaves. As expected, specialists were more efficient than generalists in exploring leaves as a dietary resource. However, the interaction between the two feeding groups could not be evaluated due to predation of *C. riparius* by *Allogamus*.

Results suggest that these fungicides can have negative effects on microbial decomposers and, at the long-term, such effects might upscale to higher trophic levels, thus compromising ecosystem functions.

Resumo

Em rios de baixa ordem, onde a luz solar que penetra as copas das árvores é insuficiente, os detritos vegetais da vegetação ribeirinha constituem a principal fonte de energia para os organismos aquáticos. A decomposição deste material é um processo chave em ecossistemas de rio. Os principais intervenientes na decomposição da vegetação alóctona são os decompositores microbianos e os invertebrados detritívoros. Os fungos, particularmente os hifomicetos aquáticos, têm um papel chave na reciclagem da matéria orgânica e constituem um elo importante nas cadeias alimentares baseadas em detritos. O uso generalizado e extensivo de compostos antifúngicos, que incluem agroquímicos e fármacos, pode afetar comunidades de fungos não-alvo. Os efeitos do tebuconazol (agroquímico), do clotrimazol e da terbinafina (fármacos) nos ecossistemas de rio foram avaliados na decomposição da folhada usando um modelo decompositor-triturador-coletor.

Numa primeira fase, os efeitos diretos dos fungicidas sobre as comunidades microbianas foram avaliados na biomassa e na esporulação dos fungos, bem como na decomposição da folhada. Para tal, submergiram-se folhas de amieiro num ribeiro não poluído, durante 14 dias, de forma a permitir a colonização por microrganismos. Posteriormente, as folhas colonizadas foram expostas, em microcosmos laboratoriais, a 8 concentrações de cada fungicida. Sob condições de controlo, foram identificadas 8 espécies de fungos, com dominância de *Articulospora tetracladia* e *Flagellospora curvula*. A exposição aos fungicidas resultou numa redução geral da diversidade e da riqueza da comunidade, sendo também observadas alterações na dominância das espécies. O tebuconazol e o clotrimazol reduziram significativamente a biomassa e a reprodução dos fungos. A terbinafina estimulou a esporulação e não provocou efeitos mensuráveis na biomassa. Contudo, nenhum dos fungicidas testados afetou as taxas de decomposição da folhada.

Na segunda experiência, os efeitos indiretos dos fungicidas foram avaliados no nível trófico seguinte (invertebrados detritívoros), avaliando a perda de massa por *taxa* especialistas (triturador, *Allogamus* sp.) ou generalistas (coletor, *Chironomus riparius*). Os discos de folhas da experiência anterior (expostos ao respetivo fungicida) foram dados aos invertebrados para avaliar se as folhas pré-expostas aos fungicidas afetavam o comportamento alimentar dos invertebrados. Em geral, a alimentação de *C. riparius* e *Allogamus* sp. não foi afetada pela exposição às folhas contaminadas pelos fungicidas. Como esperado, os especialistas revelaram-se mais eficientes que os generalistas na utilização das folhas como alimento. Contudo, não foi possível avaliar a interação entre os dois grupos funcionais devido à predação de *Allogamus* sobre *C. riparius*.

Os resultados sugerem que estes fungicidas podem ter efeitos negativos sobre os decompositores microbianos e, a longo prazo, esses efeitos podem afetar níveis tróficos superiores, comprometendo as funções do ecossistema.

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1. Introduction

1.1. The importance of biodiversity for the functioning of freshwater ecosystems

Freshwater ecosystems are essential for sustaining life, providing vital resources and conditions for a wide variety of plants, animals and microorganisms. Although freshwater resources only represent less than 1% of the Earth's surface, these ecosystems provide habitat for countless species, estimated at around 6% of the 1.8 million known species (Balian *et al.* 2008).

Human dependence on water resources is not only based on essential goods. The development of various economic, social and leisure activities are also reliant on freshwater ecosystem services, which depend on its biodiversity. However, human population growth led to an increasing demand for freshwater and the unsustainable exploitation of resources have exposed ecosystems to high anthropogenic pressure, threatening biodiversity and associated ecosystem services (Lake *et al.* 2000; Dudgeon *et al.* 2006). Loss of biodiversity and consequent changes in ecosystem services, such as provisioning, regulating, supporting and cultural services provided by biodiversity, can severely affect the environment and human well-being (MA, 2005). Shifts in the structure of biotic communities and loss of species raised concern regarding the effects of biodiversity on ecosystem functioning, hence placing the discussion on the biodiversity-ecosystem functioning relationship (BEF) as a central topic in ecology (Duffy 2008).

Pioneer investigation on biodiversity effects upon ecosystem functioning were conducted in terrestrial ecosystems, particularly in grasslands (Tilman, Lehman & Thomson 1997; Hector *et al.* 1999). Experimental evidence proposes two main processes to explain the positive effects of species diversity: sampling effect and niche complementarity. The first mechanism implies that in more diverse communities there is a greater probability of encountering dominant species, which tend to be those with higher productivity (e.g. Hector *et al.*, 1999; Huston, 1997). Loreau and Hector (2001) proposed the selection effect, a mechanism related to the sampling effect, that highlights the role of individual species traits dictating their performance in the community. According to this concept, the effects of species richness on ecosystem processes can either be negative or positive, depending on which traits are favoured within the community. Complementarity is frequently hard to differentiate from sampling effect, as both mechanisms can co-exist (Naeem 2002). In a community comprising species with complementary niches and abundant resources, the positive interactions

prevail due to facilitative interactions between species or niche differentiation, resulting in a more efficient use of the resources (Loreau & Hector 2001).

Differences in the niche breadth and niche overlap can help to overcome some of the obstacles inflicted by habitat degradation or environmental stressors. Mihuc (1997) recognizes that the presence of generalist species in the community may guarantee ecosystem functioning. Niche theory states that the effects of habitat degradation are greater in specialist species, as they benefit from homogeneous environments (space or time), contrary to generalist species that benefit from heterogeneity (Kassen 2002; Marvier, Kareiva & Neubert 2004). In theory, chaos may lead to the disappearance of specialists but the function may be assured by generalist species, which present the necessary traits to accomplish such role. Therefore, the response of a community under stress can be influenced by the number of specialist and generalist species present (Julliard *et al.* 2006).

Freshwater ecosystems exhibit unique features that constrict the direct extrapolation of insights on biodiversity-ecosystem functioning from terrestrial systems (Giller *et al.* 2004). In stream ecosystems, most studies focused on the effects of the diversity of consumers (microbial and/or invertebrate community) and resources (leaf litter) on organic matter decomposition (e.g. Kominoski, Hoellein, Leroy, Pringle, & Swan, 2010; Lecerf & Richardson, 2010; Srivastava *et al.*, 2009). This is an extremely relevant ecological process that has allowed to test some of the predictions of the biodiversity-ecosystem functioning relationship, under different environmental contexts (Fernandes, Duarte, Cássio, & Pascoal, 2013; Pascoal & Cássio, 2008; Reiss, Bailey, Cássio, Woodward, & Pascoal, 2010). It is also useful to assess the effect of anthropogenic pressures on the ecological process (decomposition) and its biological intervenients (microbes and invertebrate decomposers).

1.2. Plant-litter decomposition in freshwater ecosystems

A common feature of all ecosystems is the decomposition of organic matter, an essential process that allows the recycling of nutrients (Swift, Heal & Anderson 1979; Cadisch & Giller 1997), thus regulating the availability of nutrients and consequently the growth of organisms, upscaling to community structure (Wardle & Van der Putten 2002). In low-order forest streams, the allochthonous input of leaf litter from riparian vegetation is the main source of energy (Gessner, Chauvet, & Dobson, 1999). Commonly, these systems have limited primary production, due to shading from the surrounding canopies, low temperatures and low levels of inorganic nutrients (Benfield

1996). Gessner and colleagues (1999) characterize leaf litter decomposition in streams as an integrative process resulting from the interaction of multiple factors, including the type of allochthonous organic matter, the physical and chemical characteristics of the water and the activity of decomposing organisms. Upon reaching the stream, organic matter can be used by stream biota, stored or transported downstream, depending on stream retentiveness (Abelho 2001; Larrañaga *et al.* 2003; Elosegi 2005).

The large allochthonous organic debris that enter the watercourse, called coarse particulate organic matter (CPOM), are exposed to various stages of physical, chemical and biological transformation, resulting in several products including microbial and invertebrate biomass, fine particulate organic matter (FPOM), dissolved organic matter (DOM), inorganic nutrients and carbon dioxide (Gessner *et al.*, 1999). This breakdown can be divided in three stages: leaching of soluble compounds, microbial conditioning, and physical and biotic fragmentation (Allan & Castillo 2007). Although these stages generally occur sequentially, some temporal overlapping can also occur due to the complexity of the process (Gessner, Chauvet & Dobson 1999). Leaching is considered to be the initial decomposition step of plant litter in aquatic environments, with significant weight loss (up to 30%) within 24 hours of immersion (Petersen & Cummins 1974). The leaching of soluble compounds, such as phenols, carbohydrates and amino acids, facilitates colonization by microbial decomposers, including bacteria and fungi, increasing the carbon and nitrogen contents of the leaf (Canhoto & Graça 1996; Casas & Gessner 1999). Kaushik and Hynes (1971) concluded that, in the initial steps of organic matter decomposition, microbial activity, particularly that of fungi, are essential for the increase of leaf protein content. Microbial conditioning of plant litter, characterized by the colonization of microorganisms, contributes to degradation of the leaf material due to the mechanical and enzymatic activity driven by fungi and bacteria and enhances leaf palatability to invertebrate shredders due to nutrient enrichment of plant litter (Graça, 2001; Suberkropp, 1998). Physical and biotic fragmentation result from the abrasion enforced by water flow and the feeding activity of invertebrate shredders and microbes (Gessner *et al.*, 1999). Some authors have suggested that fungi dominate microbial decomposing activity at early stages of litter decomposition, comprising more than 90% of the total microbial biomass (Pascoal & Cássio, 2004), whereas bacteria activity increases after plant litter has been partially broken down (Baldy *et al.* 2007).

A major role in early stages of plant litter decomposition in streams is attributed to aquatic hyphomycetes (Gessner, Gulis, Kuehn, Chauvet, & Suberkropp, 2007; Suberkropp, 1998). Fallen leaves are also colonized by a variety of terrestrial fungi (Barlocher & Kendrick 1974) but their activity upon entering the stream is poorly known

and assumed to be low. Aquatic hyphomycetes, also known as Ingoldian fungi, are a phylogenetically heterogeneous group of fungi (Marvanová 1997), which sexual stage is yet to be described for most species (Shearer *et al.* 2007). Various morphological and physiological adaptations of aquatic hyphomycetes enable their colonization of several types of substrates, such as leaves and wood, in flowing waters worldwide (Gessner *et al.*, 2007). Morphological adaptations, as the production of numerous asexual spores (conidia) with tetrarradiate or sigmoid shapes, along with the production of mucilages at the ends of conidial arms, allow a more efficient attachment to substrata (Read, Moss & Jones 1992). Physiological adaptations grant them the capacity to produce a variety of extracellular enzymes, with cellulolytic, pectinolytic and proteolytic activity (Barlocher & Kendrick 1974; Suberkropp 1992), which are able to break the major plant polysaccharides.

The activity of aquatic microorganisms can constrain the entire food chain and ecosystem processes. Several studies describe the preference of invertebrate detritivores for debris colonized by decomposing microorganisms (Graça, Maltby, & Calow, 1993; Kiran, 1996). Degradation of polysaccharides and the increased nutrient content in the leaf (Barlocher & Kendrick 1974), resulting from microbial action, seem to improve the palatability of leaf material to consumers (Suberkropp 1998a). Bacteria also produce enzymes that degrade plant polysaccharides (Burns 1982), but their contribution to the process appears to be lower than that of fungi, as assessed from microbial biomass and productivity (Baldy, Chauvet, Charcosset, & Gessner, 2002; Hieber & Gessner, 2002; Pascoal & Cássio, 2004). This can be explained by their reduced or absent invasive ability, confining bacteria to the plant surface (Porter, Newell & Lingle 1989), unlike fungi.

In streams, a considerable community of benthic invertebrates usually inhabits the stream bed, also playing an important role in litter decomposition. Cummins (1973) categorized macroinvertebrates into major groups of shredders, grazers or scrapers (which feed on plants and periphyton), collectors (including collector gatherers and filter-feeders), and predators. Shredders and collectors feed on CPOM and FPOM, respectively, and therefore constitute the macroinvertebrate detritivores (Graça, 2001). Shredders display adapted mouthparts for the maceration of CPOM, that are physically converted to FPOM, making them main contributors to litter breakdown in streams (Graça, 2001). In addition, FPOM is also produced in the form of faecal pellets, constituting an important food source for collector gatherers and filter-feeders (Canhoto & Graça, 1996; Graça, 2001).

As mentioned previously, macroinvertebrate detritivores preferentially feed on conditioned leaves, due to the improvement of leaf nutritional quality (Barlocher &

Kendrick 1974; Suberkropp 1998a). The advantage of consuming conditioned leaves can be explained by either feeding directly on the microorganism, hence increasing the nutrient content per unit mass of the leaf substrate (Allan & Castillo 2007), or by eating the modified plant substrates due to the enzymatic microbial action instead of the poorly-digestible structural carbohydrates (Bärlocher 1985). Moreover, several studies demonstrate that the type of fungal species colonizing the leaves appear to affect shredder consumption (Barlocher & Kendrick, 1974; Lecerf, Dobson, Dang, & Chauvet, 2005; Suberkropp, 1984). Due to the complexity of the decomposition process and the intricate relationships among its intervenients, this important function can be affected by environmental stressors, such as those induced by humans.

1.3. Anthropogenic disturbances in freshwater ecosystems

Freshwater ecosystems are exposed worldwide to enormous pressure but possibly the most important drivers of biodiversity changes are anthropogenic factors. Human-induced habitat alterations or destruction, introduction of invasive species, overexploitation of resources and pollution lead to permanent changes in aquatic biodiversity and ecosystem functions (Dudgeon et al. 2006). The increasing demand for crop protection chemicals and pharmaceuticals, driven by human population growth, led to an increased contamination and deterioration of freshwater ecosystems worldwide. Even though industrialized countries have made substantial progress in reducing water pollution from domestic and industrial point sources, threats from excessive nutrient enrichment and other chemicals are growing (e.g. Colburn, Dumanoski & Myers, 1996). These agents of change may affect ecosystem functions via direct action upon its intervenients or by producing cumulative indirect effects.

Once pollutants reach aquatic habitats, direct toxic effects on aquatic biota can be observed and the response to these stressors varies with the intensity and duration of exposure to the toxicant. Studies in this field are frequent, in part because predictive criteria to estimate risk and to establish permissible levels of contamination are based on the response of species to contaminants (Long et al. 1995; Rohr, Kerby & Sih 2006). Typically, the direct effects of toxicants consist in a decrease of organism abundance, either by increased mortality or reduced fecundity. Direct sub-lethal effects, as physiological stress or behavioural impairment, may also be possible. Given that in a community some species exhibit a wide range of tolerance to specific toxicants, a toxicant may exert lethal or sub-lethal effects on some species, but no observable effects on others. Nevertheless, the entrance of toxicants in aquatic environments may

also bear some consequences due to indirect effects via several ecological mechanisms (Fleeger, Carman & Nisbet 2003; Rohr, Kerby & Sih 2006). Trophic cascades are a well-studied and flagrant example of an indirect effect, commonly classified as "top-down" (predator influence on lower trophic levels) or "bottom-up" (nutrient/food/prey influence on higher trophic levels) (Pace et al. 1999). Pace and collaborators (1999) defined trophic cascades as reciprocal predator-prey effects that can alter the abundance, biomass or productivity of a population or trophic level, across more than one level of a food web. A meta-analysis conducted by Shurin and colleagues (2002), combining several reports that detected trophic cascades in a diversity of ecosystems, showed that aquatic systems have stronger trophic cascades than terrestrial ecosystems. Contaminant-induced trophic cascades have been the focus of several studies. A review by Fleeger and colleagues (2003) compiled 150 papers that point to indirect toxicant effects on aquatic environments. Forrow and Maltby (2000) reported that discharges from a highway reduced the feeding rate of amphipods by reducing food quality. The contamination caused changes in the microbial community structure and growth and consequently affected food quality to amphipods. Furthermore, Duffy and Hay (2000) observed that insecticide applications had effects on non-target species. The presence of insecticide caused shifts in the macroalgal community structure that resulted from the reduced abundance of small grazers. Disturbances in higher or lower trophic levels can alter the ecosystem structure and nutrient cycling.

Agricultural and industrial exploitations are major contributors to alterations in ecosystems. These can cause soil, water and air degradation by fertilizers, pesticides and industrial spillage, which can extend to the surrounding terrestrial and aquatic systems (Stoate et al. 2009). Given the intensification of these human activities over the past decades, the occurrence of contaminants in aquatic ecosystems is frequent and raises concern about their potential impacts on the environment. As a result of continuous use and diffuse application techniques, pesticide residues, for example, can commonly be found in ground and surface waters (Berenzen et al. 2005; Bereswill et al. 2012). These compounds may persist in the environment and be toxic to non-target organisms, possibly displaying the ability to modify natural populations fitness (Zubrod, Bundschuh, Feckler, Englert, & Schulz, 2011).

1.3.1 Fungicide contaminations

Fungicides, of agricultural or pharmaceutical origin, pose a major threat to freshwater ecosystems, as they are able to alter the microbial community, hence

altering the food web structure and constraining ecosystem functions (Montuelle *et al.* 2010; Rasmussen *et al.* 2012). Research demonstrated that aquatic non-target fungi are vulnerable to fungicide contamination (Dijksterhuis *et al.* 2011; Cuco *et al.* 2017), leading to alterations in population dynamics, community composition and ecosystem functioning (Boxall, 2004; Zubrod *et al.*, 2011). Anti-fungal substances are widely used for the protection of crop plants in agriculture (fungicides) and the treatment of various fungal diseases (human and veterinary pharmaceuticals), being consequently detected in aquatic environments (e.g. Hirsch, Ternes, Haberer, & Kratz, 1999; Kahle, Buerge, Hauser, Muller, & Poiger, 2008).

The input of fungicides in freshwaters is highly dependent on the type of use. Fungicides from agriculture are transported to water courses through various pathways, including diffuse inputs from spray application, runoff and flow through tile drains (Barth *et al.* 2009), outflows from wastewater treatment plants (Gerecke *et al.* 2002) and contaminated sediments (Bermúdez-Couso *et al.* 2007). In temperate regions, the usage of these substances is highly dependent on weather conditions and during heavy precipitation events the runoff of fungicides from fields adjacent to aquatic ecosystems tends to rise (Neumann *et al.* 2002; Kronvang *et al.* 2004). Fungicides used in human and veterinary therapeutics are released to the environment through different routes (Boxall 2004). During the manufacturing process some residues can be discharged in surface waters. After administration, topically or ingested, the pharmaceutical antifungals are absorbed, metabolized and then excreted to the sewage systems and wastewater treatment plants. The physicochemical properties of the compounds determine their fate in the treated plants, where hydrophilic compounds are more likely to pass through and hydrophobic compounds are more likely to partitionate to the sewage sludge. The incomplete degradation during the wastewater treatment causes contamination in effluents (Hirsch *et al.* 1999) and the application of sewage sludge can contaminate the lands and water bodies if runoff occurs (Boxall 2004).

Fungicides constitute a chemically diverse group of compounds that attack fungi, inhibiting their development or causing their death (McGrath 2004). Nevertheless, fungicides can have distinct mechanisms of action and be classified accordingly. They can act by inhibiting sterol biosynthesis, energy production, amino acid synthesis, cell division or work within multiple sites of action (Figure 1) (Maltby, Brock & van den Brink 2009).

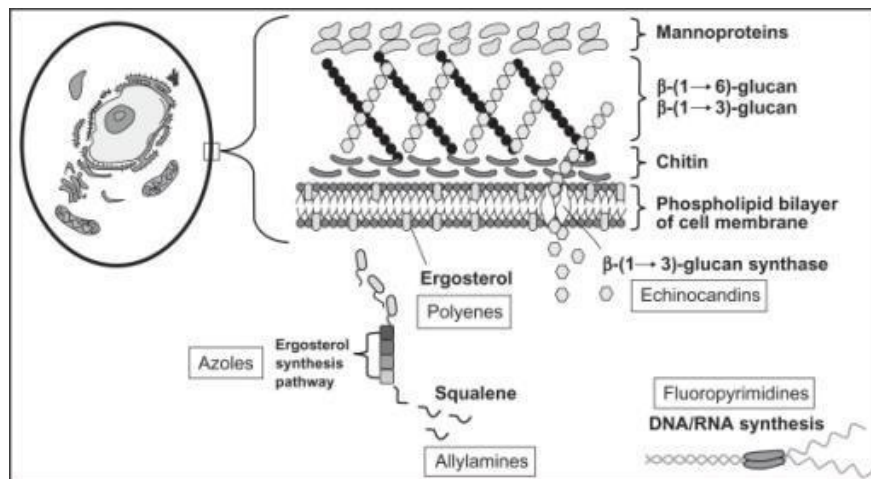


Figure 1- Targets of systemic antifungal agents. Boxes represent fungicide families according to the mode of action (polyenes, azoles, allylamines, fluoropyrimidines, and echinocandins) (source: Ashley *et al.* 2006).

Many of the most effective antifungal agents used in the medical and agrochemical fields act by inhibiting ergosterol biosynthetic pathway (Barrett-Bee & Ryder 1992). These fungicides have the ability to hamper the formation of viable spores or retard spore maturation (Fuchs & Drandarevski 1976). Since ergosterol is a key and specific constituent of fungal cell membranes, fungicides that act on this pathway turn out to be specific and less harmful to non-fungal organisms. However, recent studies demonstrated that fungicide contamination scenarios can affect aquatic non-target fungi (Dijksterhuis *et al.* 2011) and may alter population dynamics, community composition and ecosystem functioning (Bundschuh *et al.*, 2011; Zubrod *et al.*, 2011). Fungicides that inhibit ergosterol biosynthetic pathway can act at different stages (Figure 2), and azole and allylamines are among the most commonly used fungicides (Barrett-Bee & Ryder 1992).

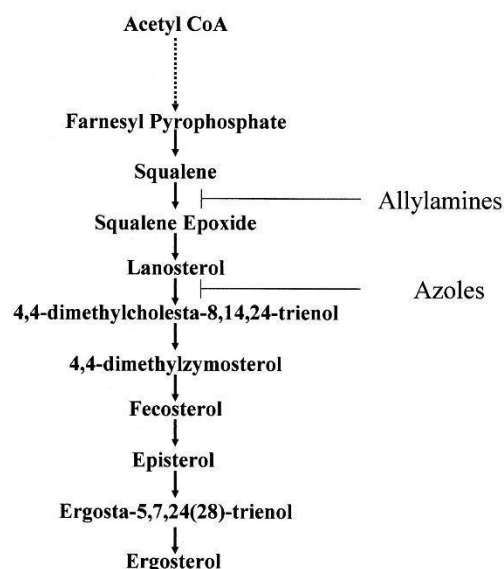


Figure 2 - Sites of action of azole and allylamine fungicides on the ergosterol biosynthesis pathway.

1.3.2 Azole fungicides

Azole fungicides represent a large group of substances widely used for the protection of crop plants and in the treatment of various fungal diseases in human and veterinary therapeutics, due to their relatively low cost and effectiveness against a wide range of fungal pathogens (Price *et al.* 2015). The first azole fungicide was reported in 1944, but it was only commercialized in 1958 as a pharmaceutical (chlormidazole) (Sheehan, Hitchcock & Sibley 1999). As agrochemicals, azoles were only introduced in the 1970's, with the commercialization of imazalil and triadimefon (Price *et al.* 2015).

Azoles act by inhibiting the lanosterol-14 α -demethylase (a cytochrome P450 enzyme, encoded by the *CYP51* gene) (Kahle *et al.* 2008), preventing the demethylation of lanosterol in ergosterol (see figure 2), which is an essential component of the fungal cytoplasmic membrane (Hof 2001). Consequently, the reduction of ergosterol formation and associated accumulation of 14 α -methyl sterols impair the cell membrane permeability and fluidity, disrupting fungal growth (Hof 2001). Azole fungicides are considered fungistatic agents rather than fungicidal (Hof 2001), although at higher concentrations some can become fungicidal (Ryder 1992). Azole fungicides can be classified as triazoles or imidazoles, according to number of nitrogen atoms in the azole ring. Triazole antifungals have a broader spectrum of applications when compared with imidazoles (Peyton, Gallagher & Hashemzadeh 2015), and the intensive use of these compounds can generate large amounts of residues that may lead to environmental contamination. As triazole fungicides are stable in the aqueous phase (FOOTPRINT, 2010) they are continuously released to aquatic environments (Kahle *et al.* 2008)

Tebuconazole (TBZ) (Figure 3) is a triazole fungicide frequently used in agriculture (Berenzen *et al.* 2005). This fungicide is regularly detected in water courses at concentrations ranging from 1 to 30 ng L⁻¹ (Kahle *et al.* 2008; Rasmussen *et al.* 2012), and in extreme runoff events was detected at concentrations up to 175– 200 mg L⁻¹ (Elsaesser & Schulz 2008).

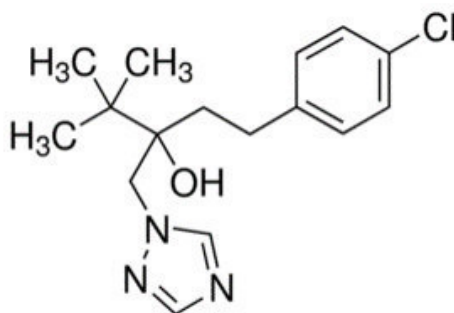


Figure 3 - Chemical structure of the fungicide Tebuconazole.

In the last decades, the presence of TBZ in streams has increased (Montuelle *et al.* 2010) which can be somehow related to the prohibition of carbendazim in fungicide formulations (EC 1107/2009). The intensive use of azole fungicides encouraged studies aiming to understand their impacts on aquatic detritivores and microbial decomposers (e.g. Bundschuh *et al.*, 2011; Rasmussen *et al.*, 2012). The effects of this fungicide on microbial communities as been assessed both in soils (Cycoń *et al.* 2006; Muñoz-Leoz *et al.* 2011) and in aquatic environments (Bundschuh *et al.*, 2011; Zubrod *et al.*, 2011). Although TBZ has a relatively short half-life in water (28 days), when compared to its soil half-live (597 days) (Kegley *et al.* 2014), the input on water courses can be constant due to the presence of this compound in soils. It is considered to be toxic to aquatic organisms and may cause negative effects in phytoplankton populations and aquatic plants, as well as long-term effects in the case of fish and zooplankton intoxication (Kegley *et al.* 2014). Studies suggest that the main aquatic microbial communities threatened are fungi. TBZ can inhibit ergosterol biosynthesis, hampering fungal mycelium growth (Copping & Hewitt 1998), causing shifts in the community composition (Bundschuh *et al.* 2011) and altering fungal activity-related processes, such as litter decomposition (Bundschuh *et al.* 2011; Rasmussen *et al.* 2012) and disease (Cuco *et al.* 2017). In a study by Artigas and colleagues (2012), leaves exposed to TBZ led to a lower fungal biomass and reduced the ability of microorganisms to decompose cellulose and hemicellulose compounds.

Clotrimazole (CTZ) (Figure 4), an imidazole fungicide, is a pharmaceutical mainly used for the treatment of dermatological and gynaecological fungal infections.

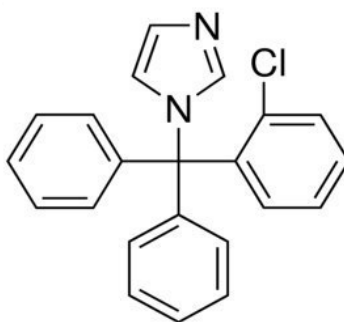


Figure 4 - Chemical structure of the fungicide Clotrimazole.

This fungicide was first synthesised in 1969, and in 2002 was included in the OSPAR List of Chemicals for Priority Action. Approximately 25 tonnes of clotrimazole are bought on the European market each year (OSPAR, 2013) and discharges from municipal wastewater treatment plants are the main source of contamination. Environmental half-life is expected to be more than 60 days, although authors have not yet reached consensus (OSPAR, 2013). In German and UK rivers this compound is regularly detected, at concentrations ranging from 3 to 54 ng L⁻¹ (Peschka, Roberts & Knepper 2007), and in UK estuarine waters it is the most detected fungicide, with concentrations fluctuating between <1 to 34 ng L⁻¹ (Roberts & Thomas 2006). Despite the widespread presence of CTZ, ecotoxicological studies have been mainly focused on algae (e.g. Porsbring, Blanck, Tjellström, & Backhaus, 2009), crustaceans and fish. Porsbring and collaborators (2009) observed that at environmentally realistic concentrations (0.017 g L⁻¹) algal demethylases were inhibited and at higher concentrations the community growth was reduced. This drug was also toxic to the marine shrimp *Palaemon serratus*, since it led to a significant reduction in survival at high concentrations (2.78 µg L⁻¹). The effect of CTZ on freshwater microorganisms is yet to be evaluated.

1.3.3 Allylamine fungicides

Allylamines are a class of synthetic antimycotics characterized functionally by their action as squalene epoxidase inhibitors (Patrany, Ryder & Stutz 1984). Naftifine was the first of these compounds to be discovered in 1974 (Georgopoulos *et al.* 1981). Allylamines act on the early steps of ergosterol biosynthesis, inhibiting the squalene epoxidase responsible for the catalysis of squalene epoxidation (see Figure 2). This results in an accumulation of squalene and a deficiency of the end-product of the pathway, ergosterol (Ryder, 1992), compromising cell permeability and leading to a

disruption of cellular organization. Contrary to azole fungicides, the primary action of allylamines is fungicidal. This ability to kill fungi contributes significantly to its therapeutic efficacy and can be overall explained by the toxic accumulation of squalene (Ryder, 1992).

Advances made during the 1990s, while trying to improve naftifine antimycotic efficacy, led to the development of terbinafine (Kauffman & Carver 1997). Terbinafine (TRF) (Figure 5) is frequently used for the treatment of a vast range of infections caused by dermatophytes and yeasts (Kauffman & Carver 1997), and several studies found it to be more effective than standard azole drugs such as clotrimazole and ketoconazole (e.g. Jones, 1990; Villars & Jones, 1989). During wastewater treatment, TRF is effectively removed from the water, however it is still present in the sludge (Lindberg, Fick & Tysklind 2010). This sludge is commonly used as fertilizer and the application on agricultural fields can impair terrestrial organisms or lead to re-entrance in water courses. In a screening of antimycotics in Swedish sewage treatment plants, Lindberg and colleagues (2010) detected TRF at concentrations oscillating between 4 and 30 $\mu\text{g kg}^{-1}$ in the digested sludge. However, data on the effects of this compound on aquatic organisms, such as freshwater microbes and invertebrates, is scarce. An investigation conducted by Palomaki (2010) suggested that TRF is very toxic to the green algae *Pseudokirchneriella subcapitata*, inhibiting growth rate and reproduction.

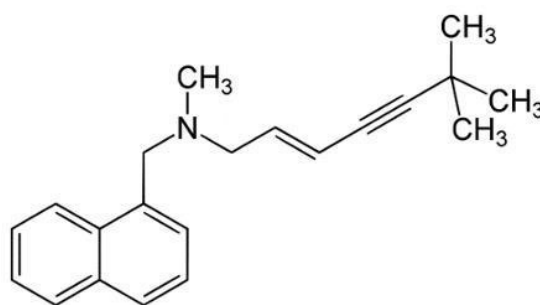


Figure 5 - Chemical structure of the fungicide Terbinafine

1.4. Decomposer-shredder-collector model as a tool for ecotoxicological assessment

Ecotoxicology emerged as a response to the numerous chemical compounds released into the environment. Concerns about the effects of these contaminants arose due to the environmental catastrophe associated with the pesticide DDT (Dichlorodiphenyltrichloroethane). The extensive use of this pesticide during the second world war caused considerable harm to wildlife populations. In 1962, Rachel Carson published "*Silent Spring*" and exposed how DDT and other pesticides had harmed animals and had contaminated the world's food supply. This publication triggered public awareness that nature was vulnerable to human intervention and was a booster for modern ecotoxicology studies (Newman 2009).

Ecotoxicology tests are used as tools to evaluate the direct effects of contaminants on single species in order to estimate permissive levels of contamination and generate data for environmental regulation (Long *et al.* 1995; Rohr, Kerby & Sih 2006). These effects are typically assessed in terms of mortality or sub-lethal endpoints, such as physiology, fecundity or behaviour (Fleeger, Carman & Nisbet 2003). However, given the complexity of natural ecosystems, analysing the dose-effect relationship between a substance and a single species might underestimate the real risk (Segner, Schmitt-Jansen & Sabater 2014). The contaminant-induced changes in species interactions, like predation or competition, or the impacts in ecological processes (such as decomposition, see above) cannot be detected with such tests, thus prompting interest on this matter (Fleeger, Carman & Nisbet 2003).

Progress in ecotoxicology protocols using populations and communities can contribute to more reliable predictions of the anthropogenic impacts on natural systems (Clements & Rohr 2009). Moreover, the decomposition process, which has been used to assess freshwaters ecological status (Pascoal *et al.*, 2001, 2003), is an ideal system for manipulative community ecotoxicology experiments, allowing the appraisal of ecosystem function. Freshwater invertebrates are extensively used in ecotoxicology experiments due to their global distribution, high reproduction rates and abundances, short life cycles and rapid adaptation to laboratorial conditions (Pradhan *et al.* 2012). In recent years, the effects of fungicides on aquatic decomposer-detritivore systems has been explored by several authors (Bundschuh *et al.*, 2011; Bundschuh, Hahn, Gessner, & Schulz, 2009; Zubrod, Baudy, Schulz, & Bundschuh, 2014; Zubrod *et al.*, 2011). Bundschuh and colleagues (2011), observed that, in the presence of tebuconazole, leaf consumption by the invertebrate *Gammarus fossarum* diminished

significantly. The results suggested that the feeding habits changed possibly due to shifts in the fungal community colonizing the leaves. The presence of this fungicide caused effects on non-target species, affected leaf processing, cascading up to invertebrate shredders.

Many studies concluded that fungicides can lead to changes in aquatic hyphomycete community (e.g. Bundschuh et al., 2011; Flores et al., 2014). Since these fungi play a crucial role in the mineralization of leaf litter and enhance leaf palatability for shredding invertebrates, depletion of these fungal populations can affect higher trophic levels. The combination of the decomposer-shredder-collector model with leaf litter decomposition can contribute to a better understanding of the indirect effects of fungicides on species interaction and ecosystem functions. Furthermore, the use of species with different degrees of specialization can help to understand if niche overlap can compensate the effects of contamination.

Trichoptera are frequently used in ecotoxicology studies (e.g. Batista, Pascoal, & Cássio, 2017; Pradhan et al., 2012). These shredders play a key role in the food chain in low-order forest streams, transferring carbon and energy from the plant material to higher trophic levels (Graça & Canhoto, 2006). The trichopteran *Allogamus* sp. (Figure 6) is a stream detritivore that belongs to the Limnephilidae family. This shredder is endemic in the Southwest Europe (Bonada *et al.* 2008) and is common in low-order streams of Northern Portugal (Varandas & Cortes 2010). In general, Limnephilidae are reported as hostile with other invertebrates, with high growth rates (Wissinger *et al.* 1996), and are functionally dominant in the decomposition process (Creed *et al.* 2009). These detritivores have a relatively long life cycle, with several aquatic stages (eggs, larvae and pupae) and an aerial phase (adult). In the larval stage, as other Trichoptera taxa, *Allogamus* sp. build a “case” as protection, from sand or plant detritus. Limnephilidae are classified as “feeding specialists” (Hering *et al.* 2009), with preference to feed on conditioned food (e.g. Arsuffi & Suberkropp, 1989; Friberg & Jacobsen, 1994).

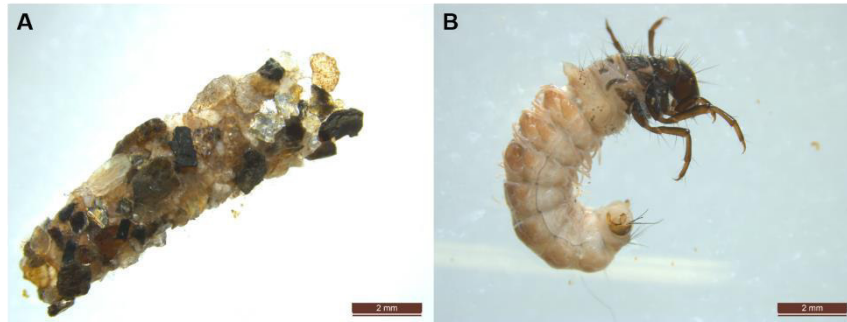


Figure 6 - Trichopteran *Allogamus* sp. (A) protection case made from sand and (B) individual without the protection case.

The dipteran *Chironomus riparius* (Figure 7) belongs to the Chironomidae family and has been largely used in toxicity assays due to its wide geographic distribution and sensitivity to environmental stress, together with its easy laboratory culture and relatively short life-cycle (Péry et al. 2003; Ha & Choi 2008). *C. riparius* is a generalist that can be found in different aquatic environments due to its ability to adapt to extreme conditions of pH, temperature, salinity and depth (Armitage, Pinder & Cranston 2012). Contrary to *Allogamus* sp., this species has a short life cycle, which includes aquatic stages (eggs, four larval stages and pupa) and an aerial (adult) phase, with the larval stage being the most enduring. It has very effective protection strategies for survival in anoxic conditions, building "chimneys" in the sediment and bearing a high level of haemoglobin capable of serving as an oxygen reservoir. Its intimate association with sediment, where contaminants can be accumulated, makes it essential to study the incidence of toxic substances in these communities as they are responsible for the transfer of energy, nutrients, and even contaminants to higher trophic levels. *C. riparius* is a collector that preferentially feeds on FPOM; however, it is capable of feeding on decaying leaves in the absence of shredders (Armitage, Pinder & Cranston 2012).

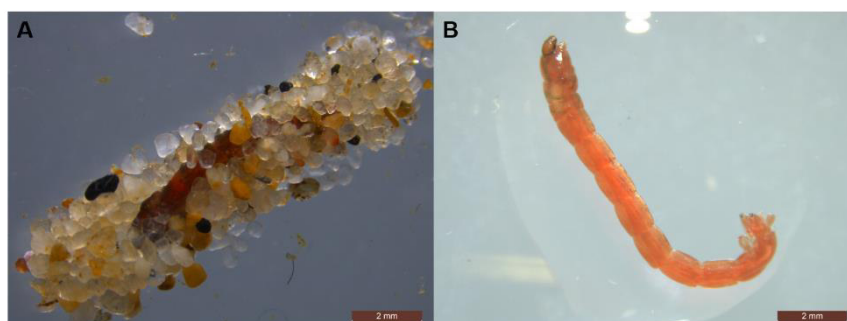


Figure 7 - Dipteran *Chironomus riparius* (A) "chimney" build with sediment and with larvae inside (B) *Chironomus riparius* 3rd instar larvae.

1.5. Objectives and outline of the thesis

The main objective of this work was to evaluate the direct and indirect effects of the fungicides tebuconazole, clotrimazole and terbinafine in freshwater ecosystems, using leaf litter decomposition and the decomposer-shredder-collector system as a model. We aimed to assess the direct and indirect effects of the fungicides by determining the contribution of each functional group to leaf decomposition under increasing concentrations of the fungicides. We expected that litter decomposition rates would decrease with increasing fungicide concentration if the contribution of each functional group to this process differs.

First, we used a microcosm approach to test the effects of increasing concentrations of tebuconazole, clotrimazole and terbinafine on aquatic microbial community and plant-litter decomposition. Measured endpoints were decomposition rate of alder leaves, and fungal biomass, sporulation rate and diversity. Subsequently, fungicide contaminated leaves were used to determine the indirect effects of the fungicides on invertebrates. To clarify if the degree of specialization of the invertebrates involved in leaf decomposition influenced their individual performance, *Chironomus riparius* and *Allogamus* sp. were chosen. The experiment was designed to discriminate the individual contribution of each invertebrate to leaf decomposition and to assess if the interaction between the organisms outcomes in an additive result or if there is facilitation/competition between the trinomial decomposer-shredder-collector.

We expected a direct negative effect of the fungicides on the aquatic microbial community and, consequently, a decrease in leaf decomposition. As invertebrates preferentially feed on conditioned leaves, we also expected a decrease in the invertebrate feeding activity in treatments with fungicide contaminated leaves.

2. Materials and Methods

2.1. Direct effects of fungicides on the activity of microbial decomposers

The direct effects of fungicides on the activity of microbial decomposers were evaluated by quantifying fungal biomass, reproduction and leaf mass loss. Leaves were used as substrate for colonization of natural microbial communities and the effects of fungicides were tested in the colonized leaves, using a microcosm approach.

2.1.1 Leaf conditioning

In the fall of 2014 and 2015, alder leaves (*Alnus glutinosa* (L.) Gaertn) were collected before abscission, dried at room temperature and stored in a dry location until use. The leaves were leached in deionized water for 8 hours and cut into 12 mm diameter discs. Sets of 60 discs were placed in fine mesh bags (0.5 mm pore size), ensuring that leaves from different years were uniformly distributed across bags. Algeriz stream, a permanent stream with little human impact and no detected sources of contamination, was chosen as the colonization site. The surrounding vegetation was of the arboreal type, where *Eucalyptus* was predominant. Depth did not exceed 30 cm and the substrate was mostly composed of sand and rocks. A total of 112 bags were submerged in the stream (41°58'76.80"N, 8°35'93.43"W) for 14 days, allowing the colonization by native microorganisms (Figure 8). In North Portugal, two weeks of leaf immersion was proven enough to attain considerable fungal biomass on alder leaves before starting a microcosm experiment (e.g. Duarte, Pascoal, Alves, Correia, & Cássio, 2010; Duarte, Pascoal, & Cássio, 2004). To estimate the initial mass of the leaves and associated microbial parameters (fungal biomass and sporulation), four leaf bags were collected after being immersed in stream water for 15 minutes.



Figure 8 - Algeriz stream, site of submersion of leaf bags to allow colonization by native microorganisms.

The physicochemical properties of the stream (temperature, pH, dissolved oxygen and conductivity) were measured *in situ* using a multiparametric field probe (Multiline F/set 3 no 400327, WTW). In the laboratory, nitrate (HACH kit, program 355), phosphate (HACH kit, program 480) and ammonium concentrations (HACH kit, program 385) were quantified, using a HACH DR/2000 photometer (Hach company, Loveland, CO, USA). Table 1 lists the physicochemical data of Algeriz stream.

Table 1- Physicochemical data of Algeriz stream in February 2016.

Parameter	Value
Temperature (°C)	12
pH	6.15
Conductivity (µS/cm)	30
Dissolved oxygen (mg L ⁻¹)	16.32
NH ₄ ⁺ (mg L ⁻¹)	<0.01
NO ₃ ⁻ -N (mg L ⁻¹)	0.44
PO ₄ ³⁻ -P (mg L ⁻¹)	0.017

2.1.2. Microcosm setup

After 14 days in the field, colonized leaf bags were retrieved from the stream and transported to the laboratory. The content of each leaf bag (60 leaf discs) was rinsed in deionized water and placed in 250 mL Erlenmeyer polypropylene flasks with

90 mL of sterilized water (see table 2 for physicochemical properties of the microcosm water, supplied by Águas do Fastio, S.A.).

The microcosms were spiked with one of three fungicides (tebuconazole, clotrimazole or terbinafine), using ethanol as a carrier (0.1% v/v final concentration). Final ethanol concentration was chosen based on a study by Moreirinha and colleagues (2011), that showed that ethanol at 0.3% v/v had no inhibition on leaf mass loss, fungal biomass, and sporulation. Tebuconazole, clotrimazole and terbinafine were obtained from Sigma Aldrich (Munich, Germany) (see Table 3). After a thorough analysis of literature, potentially toxic concentrations were chosen taking into account the occurrence of the toxicants in natural ecosystems and their chemical properties (Table 3). A geometric series of 8 concentrations was used per fungicide: 10, 20, 40, 80, 160, 320, 640, 1280 $\mu\text{g L}^{-1}$. Two negative controls (no fungicide) were also carried out, one without (CTL) and one with ethanol (EtOH) at the same concentration used in the fungicide solutions. Four replicates per concentration were used.

Microcosms were incubated on a shaker (140 rpm), at $18 \pm 1^\circ\text{C}$, under permanent artificial light, and test solutions were changed every 6 days, following procedures implemented in previous studies (e.g. Pascoal, Cássio, Nikolcheva, & Bärlocher, 2010). The experiment was carried out for 26 days, until c.a. 50% of leaf decomposition in control microcosms was observed. All discarded solutions were stored in plastic bottles to evaluate fungal reproduction (conidial production) and, at the end of the experiment, remaining leaf discs were stored at 4°C to assess leaf dry mass and fungal biomass, as described below.

Table 2- Physicochemical properties of the microcosm water (natural spring water supplied by Águas do Fastio, S.A.)

Parameter	Mean \pm SD
Dry residue (at 180°C) (mg L^{-1})	$34,0 \pm 4$
Silica (mg L^{-1})	$9,6 \pm 2$
pH (at 18°C)	6,0
HCO_3^- (mg L^{-1})	$8,0 \pm 0,8$
Cl^- (mg L^{-1})	$4,2 \pm 0,4$
SO_4^{2-} (mg L^{-1})	$1,0 \pm 0,2$
Na^+ (mg L^{-1})	$4,1 \pm 0,4$
Ca^{2+} (mg L^{-1})	$1,3 \pm 0,3$
K^+ (mg L^{-1})	$0,6 \pm 0,1$

Table 3- Properties of the fungicides tebuconazole, clotrimazole and terbinafine.

Name	CAS Number	IUPAC Name	Molecular formula	Water solubility
Tebuconazole	107534-96-3	1-(4-chlorophenyl)-4,4-dimethyl-3-(1,2,4-triazol-1-ylmethyl)pentan-3-ol	C ₁₆ H ₂₂ ClN ₃ O	36 mg L ⁻¹
Clotrimazole	23593-75-1	1-[(2-chlorophenyl)-diphenylmethyl]imidazole	C ₂₂ H ₁₇ ClN ₂	29.84 mg mL ⁻¹
Terbinafine	91161-71-6	(E)-N,6,6-trimethyl-N-(naphthalen-1-ylmethyl)hept-2-en-4-yn-1-amine	C ₂₁ H ₂₅ N	0.000738 mg mL ⁻¹

2.1.3. Leaf mass loss, fungal biomass and sporulation

A subset of 48 leaf discs from each microcosm was lyophilized during 48 h and weighed to the nearest 0.0001 mg. Initial leaf mass (dry, lyophilized) was also determined using a subsample of leaf discs collected after 15 minutes of exposure in the stream (see 2.1.1). Percentage of leaf mass loss (ml) was estimated, calculating the difference between initial and final weight ($m_i - m_f = ml$). Rates of leaf litter breakdown were assessed using the negative exponential model $mt = m0 \times e^{-kt}$, where mt is the leaf mass remaining at time t , $m0$ is the initial leaf mass, k is the rate of leaf decomposition, and e is the base of natural logarithm (Webster and Benfield 1986). Quantification of fungal biomass in leaf discs was accomplished by measuring a fungal-specific cellular constituent that occurs at constant amounts in viable mycelium: ergosterol. This is a major component of fungal membranes, and one that is commonly used to estimate fungal biomass associated with decomposing leaf litter (Gessner, 2005). Fungal hyphae are able to penetrate leaf substrates, making it hard to separate them from leaves (Gessner & Newell, 2002; Newell, 1992); hence, there is a need for a fungi-specific marker which quantification can unequivocally indicate the presence of fungi in the decomposing leaf mass. The use of ergosterol quantification presents several advantages (in comparison with chitin quantification, for example): it is the major sterol in most eumycotic fungi and is absent from vascular plants and most animals, well distinguished spectrophotometrically from plant sterols and it is likely to be rapidly degraded upon cell death allowing to estimate the living fungal biomass (Gessner & Newell, 2002). Ergosterol quantification combines sterol extraction followed by the purification of the crude lipid. For lipid extraction, sets of 6 freeze-dried leaf discs from each replicate microcosm were placed in 0.8 % of KOH/methanol solution and heated (80 °C for 30 minutes). Ergosterol was purified by solid-phase extraction (SPE) and quantified by high performance liquid chromatography (HPLC).

Spores suspended in water taken from microcosms (~250 mL) were preserved with 2 mL of 37 % formaldehyde and 100 µL of 15 % Triton X-100, to prevent their germination and adherence to the walls of the bottles where they were stored. For conidial identification and enumeration, an appropriate volume of each sample of conidial suspensions (volume was adjusted according to spore density) was filtered through a membrane (Millipore 0.45 µm pore size) and the spores retained on the membrane were stained with cotton blue in lactic acid (Bärlocher, 2005; Gessner, Bärlocher, & Chauvet, 2003). To assess fungal community composition and sporulation rates, at least 300 spores were identified and counted per experimental unit under an optical microscope (400x magnification), following the illustrated guide by Gulis and collaborators (2005) and several papers on the taxonomy of specific taxa.

2.2. Indirect effects of fungicides in generalist and specialist invertebrates

The indirect effects of fungicides on the next trophic level (detritivore invertebrates) were evaluated by assessing invertebrate biomass and non-microbial leaf decomposition. Leaf discs from the previous experiment (colonized and exposed to the respective fungicide – see 2.1) were given to the invertebrates to assess if the pre-exposure of the leaves and associated microbiota to the tested fungicides affected the feeding behaviour of invertebrates.

2.2.1 Test organisms

A culture of *Chironomus riparius* was established at the Biology Department, University of Minho, using egg masses obtained from IBED (Institute for Biodiversity and Ecosystem Dynamics), Department of Aquatic Environmental Ecology, University of Amsterdam (The Netherlands).

The culture unit was an enclosed transparent plastic box (53.5 x 41 x 34 cm) containing all the apparatus necessary to allow completion of the life cycle of the chironomids, including copulation of emerged adults (which requires a minimum of 30 cm in all three dimensions; OCDE, 2000) (Figure 9).



Figure 9 - Enclosed transparent plastic box containing all the apparatus necessary to allow completion of the life cycle of *C. riparius*.

Cultures were maintained at $18 \pm 1^\circ\text{C}$, with a 14:10 h light:dark cycle, including a 90 minute period of dawn and dusk. At the start of a new culture, approximately 150 first-instar larvae were introduced into plastic beakers (1 L capacity) containing a semi-synthetic culture medium (hardwater medium - Table 4, diluted 33% with natural mineral water – Table 2) and sea sand. Sea sand was previously sieved ($< 855 \mu\text{m}$), washed and autoclaved. Each beaker was aerated, and a suspension of Tetramin (Tetrawerke, Germany) was used as the only food source, minced to small particles and suspended in culture water before being added at a ration of 1 g Tetramin/beaker, every 2 days (Soares *et al.* 2005). After ten days, larvae were transferred to new culture beakers with fresh medium, food and sand until emergence occurred. To feed the adults, a small flask with paper impregnated in a saturated sucrose solution was placed inside the culture unit. A glass container filled with deionised water was also provided to allow oviposition. Freshly laid egg masses were transferred to small Petri dishes with culture medium until hatching occurred and the newborn larvae (1st instar) were then used to start a new culture.

Table 4- Semi-synthetic culture medium (diluted 33% with natural mineral water – Table 2)

Parameter	Concentration (mg L^{-1})
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	110.28
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	110.91
NaHCO_3	126.00
KCl	7.45

Early-stage larvae of caddisfly *Allogamus sp.* with similar size were collected in the upper reach of the Cávado river (41°48'39.1"N 7°51'44.7"W) on the 30th of December 2016, and transported to the laboratory in plastic containers with stream water. The location was chosen according to the local good water quality and diverse community of invertebrates (Pascoal et al., 2001). In the laboratory, the animals were kept in aquariums with stream water mixed with Fastio water at 1:3 proportion (physicochemical properties on Table 2), at 18±1 °C with aeration and a 14:10 hour light:dark cycle, including a 90 minute period of dawn and dusk. The animals were allowed to feed on alder leaves for 13 days prior to the experiment.

2.2.2 Feeding experimente

Different treatments were designed to determine the indirect effects of the fungicides on the feeding behaviour of the invertebrates. In total, experimental design was divided into three treatments: C – four *C. riparius* individuals; T- four *Allogamus sp.* individuals; and C+T- two *C. riparius* and two *Allogamus sp.* individuals (Figure 10). Each treatment was exposed to pre-contaminated leaves (from the previous experiment) at different concentrations of fungicide (20 and 160 µg L⁻¹) and ethanol, which was used as control. The activity of microbial decomposers in the previous experiment determined the chosen concentrations, also considering the limits of detection in the natural ecosystems. Highest concentration of TBZ found in aquatic environments was 200 µg L⁻¹ (Elsaesser & Schulz 2008), however, given the lack of data concerning both environmental detection and ecosystem effects of CTZ and TRF, same concentrations as TBZ were chosen for this fungicides.

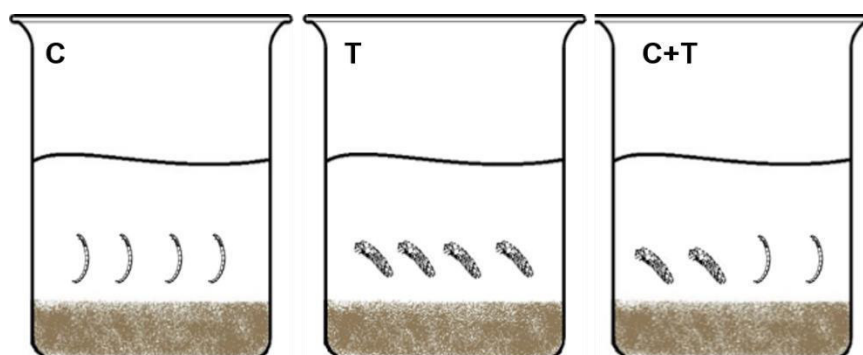


Figure 10 - Experimental design divided into three treatments: C - *Chironomus riparius* individuals, T – *Allogamus sp.* individuals and C+T - *Chironomus riparius* and *Allogamus sp.* individuals. Each treatment was exposed to leaf discs contaminated with one of the the three fungicides (tebuconazole, clotrimazole or terbinafine) at 20 and 160 µg L⁻¹. Leaves contaminated with ethanol were used in the control treatment. All leaf discs were retrieved from the previous experiment (direct effects on microbial decomposers).

Prior to initiating the feeding experiment, animals were fasted for 1 day to ensure that all individuals used in the experiment were in the same metabolic state. Alder leaves, used in the previous microbial experiment, were used as food source. To estimate initial leaf weight, leaves were lyophilized for 24 hours and weighed to the nearest 0.0001 mg.

Animals were allocated to plastic beakers, containing a 1-cm-deep layer of sea sand (same as Chironomid cultures), 100 mL of Fastio water, 14 leaf discs (spiked with the fungicides or controls) and 4 animals. All flasks were aerated with constant air flow and incubated at 18 ± 1 °C, with a 14:10 hour light:dark cycle, including a 90 minute period of dawn and dusk (Figure 11). The experiment was continued for 5 days until leaf decomposition was visible in the control microcosms.



Figure 11 - Feeding experiment setup.

At the end of the feeding experiment, CPOM was determined. Leaf consumption was calculated from the differences between initial and final (CPOM) dry weight of leaf discs. To normalize the data, leaf consumption was estimated taking into account the animal dry weight.

2.3. Statistical analysis

One-way analysis of variance (One-Way ANOVA) was used to test the effect of the concentrations of the fungicides on leaf decomposition, and fungal sporulation and biomass. Significant differences between ethanol and fungicide concentrations were analyzed using Dunnett's post-hoc test. Differences were considered significant at $p \leq 0.05$ (Zar 1996). Each fungicide was analyzed separately.

A Principal Component Analysis (PCA) was performed to ordinate the experimental units according to the fungal community composition (based on spore counts). Hellinger transformation was performed to better suit species data, giving low weights to variables with low values and many zeros. Additionally, Shannon index (H') and Pielou's evenness index (J') were used to assess the diversity of aquatic fungi according to the following equations:

$$H' = - \sum_{i=1}^S P_i (\ln P_i)$$

$$J' = H' / \ln S$$

where P_i is the relative abundance of conidia of taxon (i) and S is the total number of sporulating taxa (Legendre & Legendre 1998).

Two-way analysis of variance (Two-way ANOVA) was used to test if the concentration of each fungicide and the invertebrate treatment (C, T, C+T) affected the leaf decomposition. Significant differences between invertebrate treatments were analysed with Tukey's post-hoc test and differences between ethanol and fungicide concentrations were analysed with Dunnett's post-hoc tests. Differences were considered significant at $p \leq 0.05$ (Zar 1996). Each fungicide was analysed separately.

Univariate analyses were performed with GraphPad Prism 6.01 for Windows (GraphPad software Inc., San Diego) and multivariate analyses with R software (R Foundation for Statistical Computing, Vienna).

3. Results

3.1. Tebuconazole

3.1.1 Direct effects of tebuconazole on the activity of microbial decomposers

After 26 days of exposure, significant changes were caused by tebuconazole in the decomposition of alder leaves inoculated with stream fungi, fungal biomass associated with the leaves, sporulation rates of aquatic hyphomycetes and microbial community indices (diversity and evenness) – see Table 5. Post-hoc analyses showed that, for decomposition rates (Figure 12A) and leaf decomposition (Figure 12B), differences were only found between the control (CTL) and control with ethanol (EtOH), demonstrating the absence of direct effects of tebuconazole on leaf decomposition (Dunnett test, $P \geq 0.05$). However, tebuconazole significantly affected the other parameters, when using the EtOH control as a reference (see below).

Fungal biomass was 158 and 265 μg ergosterol per g of leaf mass in the CTL and EtOH controls, respectively (Figure 12C). The presence of TBZ significantly affected fungal biomass (Dunnett test, $P \leq 0.05$), with a decrease of 31-57% from the EtOH control from the lowest concentration upwards (except for 40 $\mu\text{g L}^{-1}$). Sporulation rates of aquatic hyphomycetes was 3.69×10^5 spores per g of leaf mass per day in the CTL, and attained 7.93×10^5 spores per g of leaf mass per day in EtOH control (Figure 12D). The exposure to TBZ significantly inhibited fungal sporulation, except at the lowest concentration (Dunnett test, $P \leq 0.05$).

Table 5 - Summary of one-way ANOVAs on the effects of tebuconazole on decomposition rates, leaf mass loss, fungal biomass, sporulation rate and Shannon and Pielou indices.

Parameter	Effect	SS	DF	MS	F	P
Decomposition rates	Concentrations	0,002	9	0,0003	8,88	< 0,0001
	Residuals	0,0008	30	0,00003		
Leaf mass loss	Concentrations	0,03	9	0,0033	6,46	< 0,0001
	Residuals	0,0155	30	0,0005		
Fungal biomass	Concentrations	63476	9	7053	4,28	0,0037
	Residuals	31302	19	1647		
Sporulation rate	Concentrations	$2,35 \times 10^{12}$	9	$2,61 \times 10^{11}$	42,48	< 0,0001
	Residuals	$1,84 \times 10^{11}$	30	$6,14 \times 10^9$		
Shannon diversity index	Concentrations	9,761	9	1,085	3857	< 0,0001
	Residuals	0,0084	30	0,0003		
Pielou evenness index	Concentrations	2,608	9	0,2898	1267	< 0,0001
	Residuals	0,0069	30	0,0003		

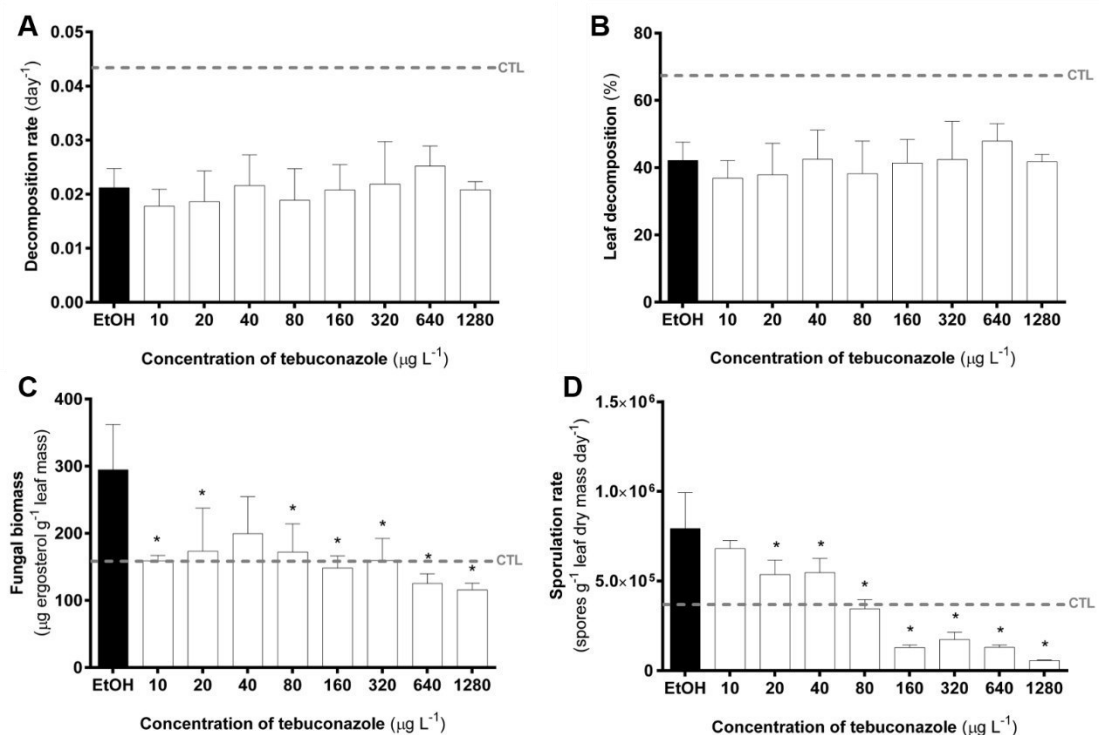


Figure 12 - Effect of tebuconazole on (A) decomposition rates of alder leaves, (B) percentage of leaf decomposition, (C) fungal biomass and (D) fungal sporulation after 26 days of exposure in microcosm. Asterisks (*) represent treatments that differ significantly from EtOH control (one-way ANOVA followed by Dunnett test, $P \leq 0.05$). Data are shown as mean \pm SD, $n=4$. Dashed grey line represents the negative control (CTL).

The diversity and evenness of aquatic hyphomycete community were significantly affected by the presence of TBZ (Dunnett test, $P \leq 0.05$; Figure 13). Shannon's index scored 1.65 and 0.90 in the CTL and EtOH controls, respectively, and significantly decreased from 10 $\mu\text{g L}^{-1}$ upwards (Figure 13A). Pielou's evenness followed the same trend, with higher values found in the controls (0.83 for CTL and 0.49 for EtOH), and then decreasing monotonically under tebuconazole stress (except for the treatment at 40 $\mu\text{g L}^{-1}$) (Figure 13B).

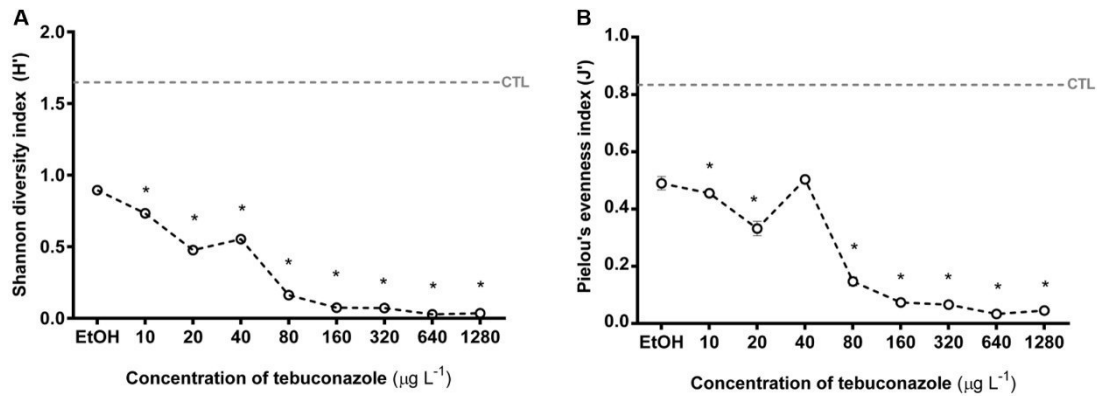


Figure 13 - Effect of tebuconazole on (A) Shannon's diversity index (H') and (B) Pielou's evenness index (J') of aquatic hyphomycete community on decomposing alder leaves (based on spore counts), after 26 days of exposure to increasing concentrations of tebuconazole in microcosm. Asterisks (*) represent treatments that differ significantly from EtOH control (one-way ANOVA, Dunnett test, $P \leq 0.05$). Data are shown as mean \pm SD, $n=4$. Dashed grey line represents the negative control (CTL).

Based on conidial morphology, a total of 8 aquatic hyphomycete species were identified on decomposing leaves after 26 days in CTL microcosms (Figure 14). The exposure to ethanol decreased species richness to 6 taxa and caused shifts in the species contributions to total conidial production. In CTL control, *Flagellospora curvula* (29%), *Articulospora tetracladia* (20%) and *Infundibura* sp. (24%) were the dominant species, whereas in the EtOH control *Infundibura* sp. (61%) and *Dimorphospora follicola* (31%) were the dominant species. In the presence of TBZ, the community structure changed, as the conidial production of *Infundibura* sp. largely increased, contributing with more than 99% to the total conidium production at the higher concentrations. This was accompanied by a decrease of other taxa and explains the decreasing pattern observed for evenness and diversity (see above).

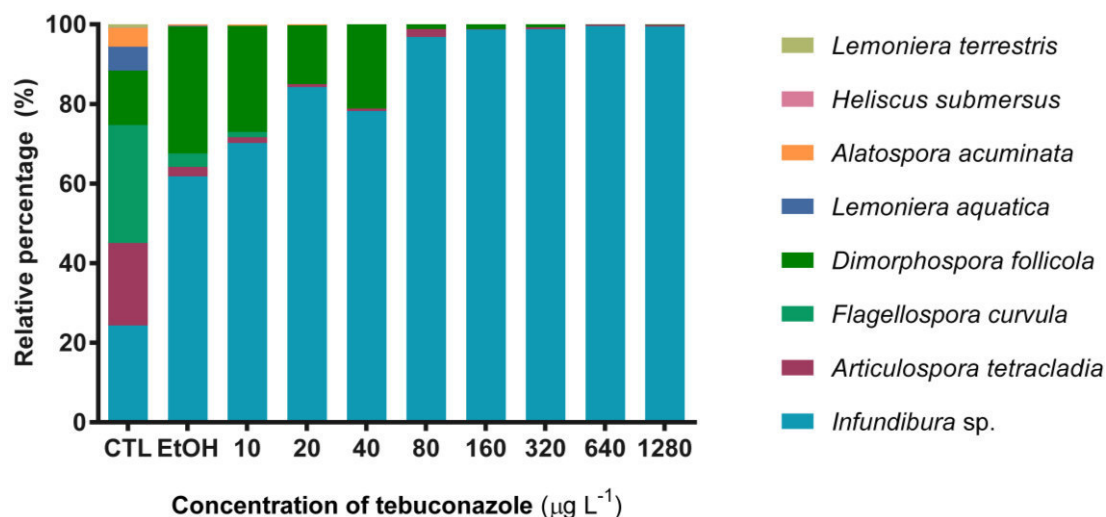


Figure 14 - Effect of tebuconazole on the relative contribution of each species to the total conidial production after 26 days of exposure in microcosms. Relative percentage was calculated as the pool of four replicates.

Principal component analysis (PCA) ordination of the eight concentrations and EtOH, according to sporulation profiles of aquatic fungi (Figure 15), showed that axes 1 and 2 explained 93.6% and 4.4% of the total variance, respectively. The first PC axis discriminated lower (EtOH and 10–40 $\mu\text{g L}^{-1}$) from higher tebuconazole concentrations (80–1280 $\mu\text{g L}^{-1}$). The ordination plots show that the separation of scores along the first axis represents a combination of increased importance of *Infundibura* sp. and decreased importance of *Flagellospora curvula*, *Dimorphospora follicola*, and – to a lesser extent – of other less abundant taxa (*Articulospora tetracladia*, *Lemoniera aquatica*, *Alatospora acuminata*, *Heliscus submersus* and *Dimorphospora follicola*) along the tebuconazole gradient. As the concentration of TBZ increased, the importance of *Infundibura* sp. increased as the other taxa become less abundant (as seen in Figure 14). Although there is some variation along the PC2 (e.g., EtOH has lower scores than the other treatments), this axis is much less important than the PC1 in discriminating the treatments.

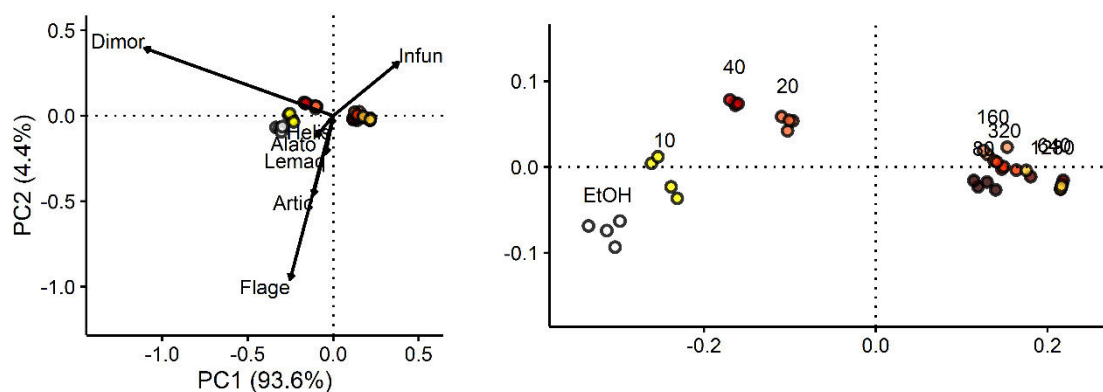


Figure 15 - Ordination plots (PCA on Hellinger-transformed data) of sporulation profiles of aquatic fungi under increasing tebuconazole concentrations, plus a negative control (EtOH). Left panel represents biplot of sample (i.e., experimental units) scores (circles) and species (arrows); right panel represents a zoom of the sample scores, identified by corresponding label near the average score for each experimental treatment. Circles are coloured using a light-to-dark gradient that corresponds to the concentration gradient of each toxicant; EtOH is represented by white circles. Species abbreviations: Flage – *Flagellospora curvula*, Infun – *Infundibura* sp., Artic – *Articulospora tetracladia*, Dimor – *Dimorphospora follicola*, Lemaq – *Lemoniera aquatica*, Alato – *Alatospora acuminata* and Helis – *Heliscus submersus*.

3.1.2 Indirect effects of tebuconazole on generalist and specialised invertebrates

The lowest TBZ concentration with a significant difference in sporulation rate ($20 \mu\text{g L}^{-1}$) and the highest ecologically relevant TBZ concentration ($160 \mu\text{g L}^{-1}$) were selected to assess the non-microbial leaf consumption rate, in three different combinations of detritivore invertebrates (Figure 16). After 5 days, the leaf consumption rate in the EtOH control was 0.614, 0.766 and 0.861 g of leaf per g of animal per day for the C, T and C+T treatments, respectively.

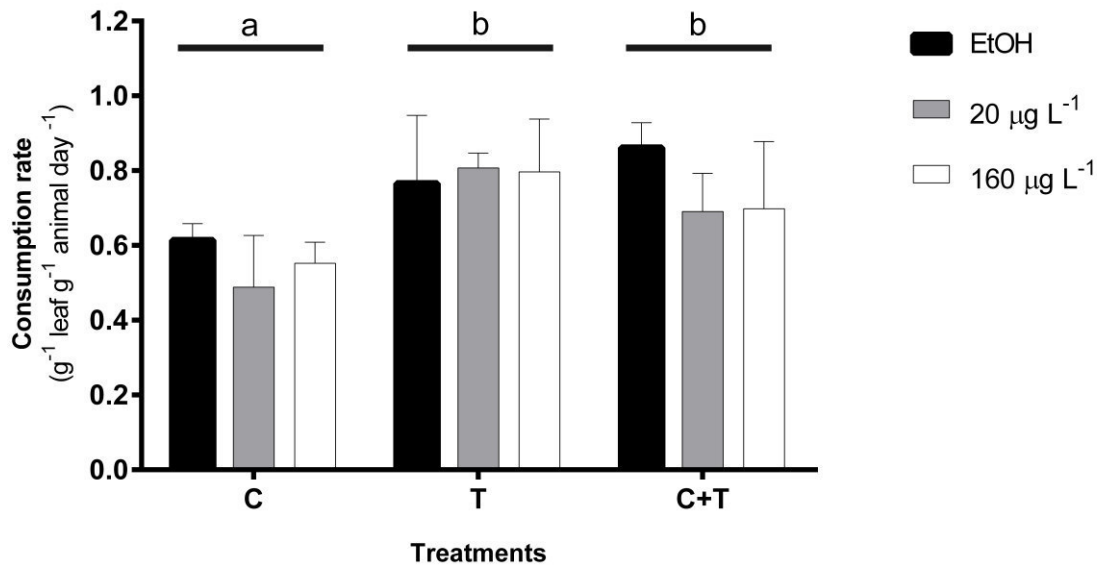


Figure 16 - Consumption rate of alder leaves (previously conditioned with microbiota and exposed to tebuconazole) by *Chironomus riparius* (C), *Allogamus* sp. (T) and both (C+T), after 5 days of exposure in microcosms. Bar colour represents different tebuconazole levels, including a negative control without addition of tebuconazole, but with added carrier (EtOH). Data are shown as mean \pm SD, $n=4$. Different letters (a, b) represent overall differences across invertebrate treatments (C, T, C+T). Asterisks (*) stand for significant differences relatively to EtOH (Dunnett's test, $P < 0.05$).

The combination of invertebrate species significantly affected leaf consumption rate (two-way ANOVA: $MS_{\text{treatment}} = 0.20$, $F_{2, 27} = 14.0$, $P < 0.0001$). On the contrary, fungicide concentration had no effect on leaf consumption rate (two-way ANOVA: $MS_{\text{concentration}} = 0.024$, $F_{2, 27} = 1.68$, $P = 0.205$), irrespective of the treatment level (no significant interaction, $MS_{\text{interaction}} = 0.016$, $F_{4, 27} = 1.12$, $P = 0.370$). Leaf consumption was significantly higher in the treatments where *Allogamus* sp. was present (Tukey test, $P \leq 0.05$, Figure 16) when compared to treatment C. However, at the initial stage of the experiment, *Allogamus* sp. preyed on *C. riparius*, not allowing a proper evaluation of the effect of both species on leaf consumption in the C+T treatment.

3.2. Clotrimazole

3.2.1 Direct effects of clotrimazole on the activity of microbial decomposers

Exposure to clotrimazole, for 26 days, caused significant changes in the decomposition of alder leaves inoculated with stream fungi, fungal biomass associated with the leaves, sporulation rates of aquatic hyphomycetes and microbial community indices (diversity and evenness) – see Table 6. Post-hoc analyses showed that, for decomposition rates (Figure 17A) and leaf decomposition (Figure 17B), differences were only found between the CTL and EtOH, demonstrating the absence of effects of clotrimazole in these parameters (Dunnett test, $P \geq 0.05$). Nevertheless, clotrimazole significantly affected the other parameters, when using the EtOH as a reference (see below).

Fungal biomass (Figure 17C) was 158 and 265 μg ergosterol per g of leaf mass in the CTL and EtOH controls, respectively. The presence of CTZ significantly affected fungal biomass (Dunnett test, $P \leq 0.05$; Figure 17C), with decreases ranging from 7.4 % (160 $\mu\text{g L}^{-1}$) to 67.7% (1280 $\mu\text{g L}^{-1}$) when compared to EtOH control.

Sporulation rates of aquatic hyphomycetes (Figure 17D) was 3.69×10^5 spores per g of leaf mass per day in the CTL, and attained 7.93×10^5 spores per g of leaf mass per day in EtOH control. The exposure to CTZ significantly inhibited fungal sporulation (Dunnett test, $P \leq 0.05$). Sporulation rate was significantly higher at the lowest concentration of CTZ, reaching 1.13×10^6 spores per g of leaf mass per day. The two higher concentrations used (640 and 1280 $\mu\text{g L}^{-1}$) significantly decreased the sporulation rate to 0.48×10^6 and 0.19×10^6 spores per g of leaf mass per day, respectively.

Table 6 - Summary of one-way ANOVAs on the effects of tebuconazole on decomposition rates, leaf mass loss, fungal biomass, sporulation rate and Shannon and Pielou indices.

Parameter	Effect	SS	DF	MS	F	P
Decomposition rates	Concentrations	0,002	9	0,0002	10,41	< 0,0001
	Residuals	0,0006	30	0,00002		
Leaf mass loss	Concentrations	0,0278	9	0,003	7,24	< 0,0001
	Residuals	0,0128	30	0,0004		
Fungal biomass	Concentrations	115007	9	12779	4,24	0,0034
	Residuals	60271	20	3014		
Sporulation rate	Concentrations	$3,04 \times 10^{12}$	9	$3,38 \times 10^{11}$	15,06	< 0,0001
	Residuals	$6,73 \times 10^{11}$	30	$2,24 \times 10^{10}$		
Shannon diversity index	Concentrations	5,667	9	0,6297	964,4	< 0,0001
	Residuals	0,0196	30	0,0007		
Pielou evenness index	Concentrations	1,173	9	0,1304	195	< 0,0001
	Residuals	0,02	30	0,000668		

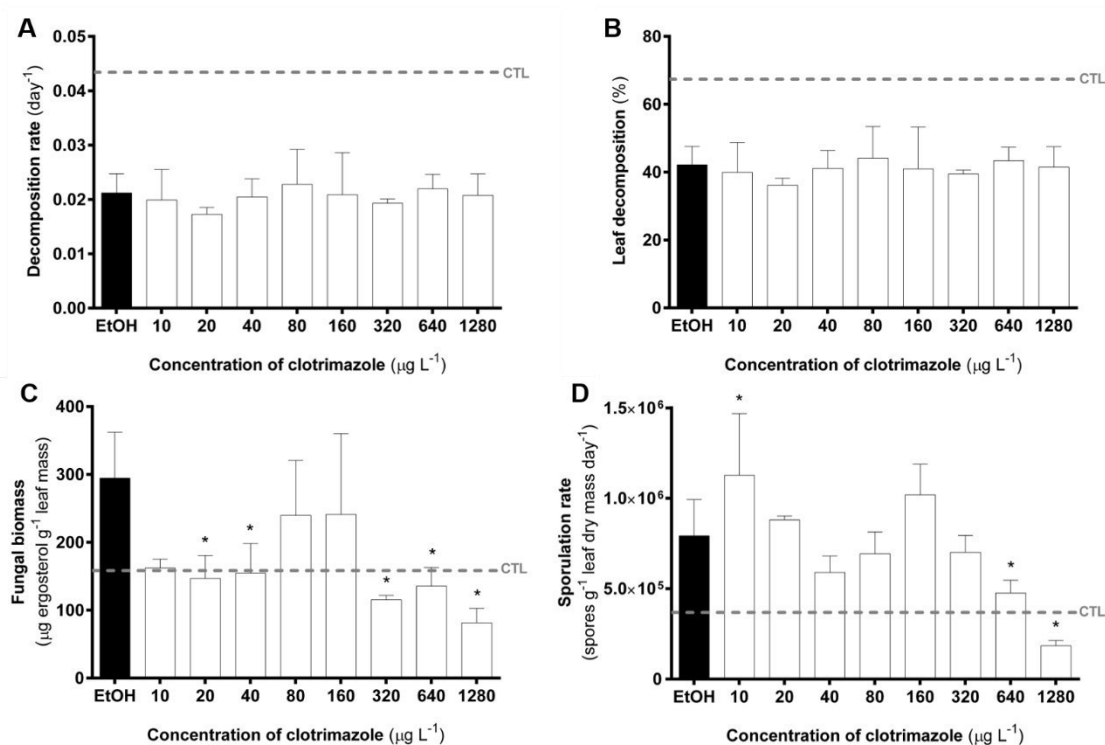


Figure 17 - Effect of clotrimazole on (A) decomposition rates of alder leaves, (B) percentage of leaf decomposition, (C) fungal biomass and (D) fungal sporulation after 26 days of exposure in microcosm. Asterisks (*) represent treatments that differ significantly from EtOH control (one-way ANOVA followed by Dunnett test, $P \leq 0.05$). Data are shown as mean \pm SD, $n=4$. Dashed grey line represents the negative control (CTL).

The diversity and evenness of the aquatic hyphomycete community were significantly affected by the presence of CTZ (Dunnett test, $P \leq 0.05$; Figure 18). Shannon's index (Figure 18A) scored 1.65 and 0.90 in the CTL and EtOH controls, respectively, with the lowest value observed at 20 $\mu\text{g L}^{-1}$ (0.32). Pielou's evenness (Figure 18B) controls CTL and EtOH scored 0.83 and 0.49, respectively. Lower values were found at lower concentrations of CTZ (10 $\mu\text{g L}^{-1}$ - 0.30 and 20 $\mu\text{g L}^{-1}$ - 0.23) and the higher value at 80 $\mu\text{g L}^{-1}$ (0.71).

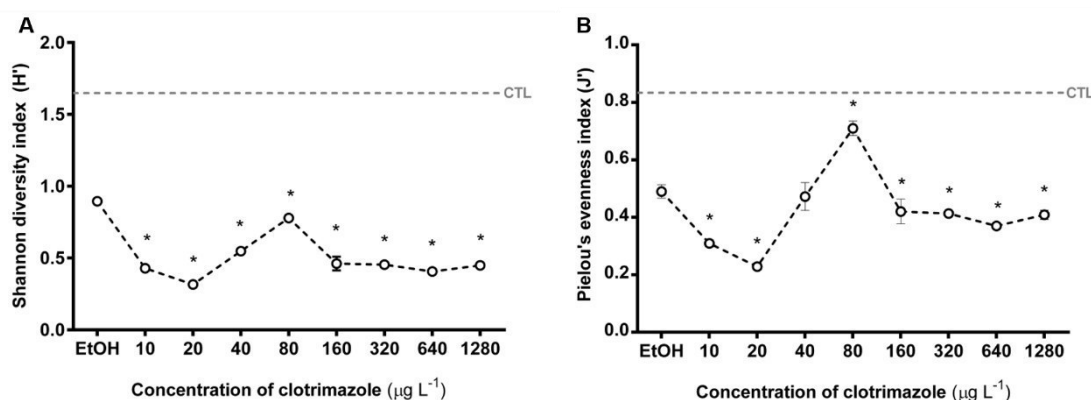


Figure 18 - Effect of clotrimazole on (A) Shannon's diversity index (H') and (B) Pielou's evenness index (J') of aquatic hyphomycete community on decomposing alder leaves (based on spore counts), after 26 days of exposure to increasing concentrations of clotrimazole in microcosm. Asterisks (*) represent treatments that differ significantly from EtOH control (one-way ANOVA, Dunnett test, $P \leq 0.05$). Data are shown as mean \pm SD, $n=4$. Dashed grey line represents the negative control (CTL).

In the CTL, a total of 8 aquatic hyphomycete species were identified on decomposing leaves after 26 days in microcosms (Figure 19). Exposure to ethanol decreased species richness to 6 taxa and triggered alterations in their contributions to conidial production. In CTL, *Flagellospora curvula* (29%), *Articulospora tetracladia* (20%) and *Infundibura* sp. (24%) were the dominant species, whereas in the EtOH control *Infundibura* sp. (61%) and *Dimorphospora follicola* (31%) were the dominant species. In the presence of CTZ, the community structure changed, as the conidial production of *Infundibura* sp. largely increased (accompanied by the decrease in the other taxa), representing more than 85% of the total conidium production at higher concentrations, while *Dimorphospora follicola* contribution diminished to 10%. These findings explain the decreasing pattern observed for evenness and diversity (see above).

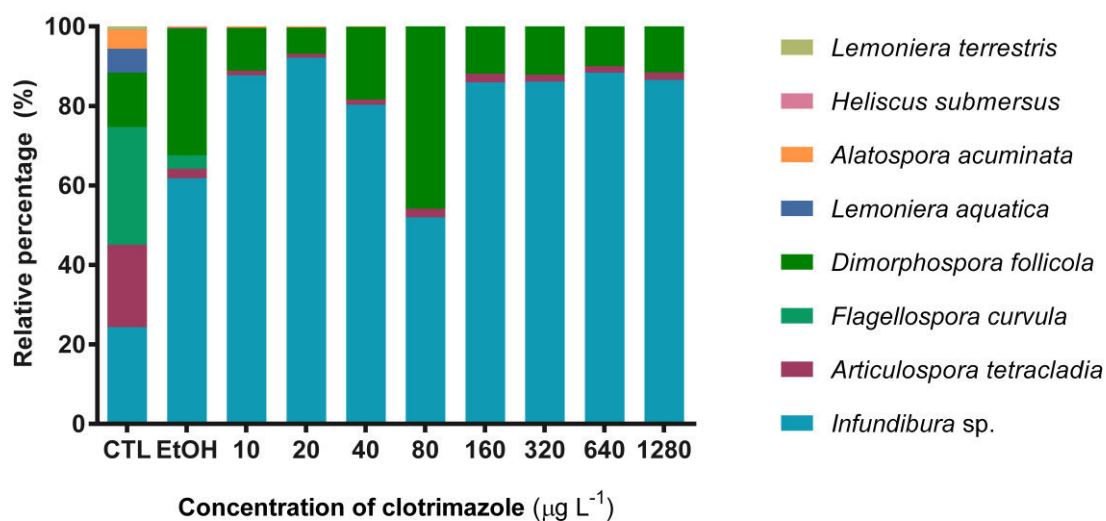


Figure 19 - Effect of clotrimazole on the relative contribution of each species to the total conidial production after 26 days of exposure in microcosms. Relative percentage was calculated as the pool of four replicates.

Principal component analysis (PCA) ordination of the eight CTZ concentrations and the ethanol control, according to sporulation profiles of aquatic fungi (Figure 20), showed that axes 1 and 2 explained 85.4% and 10.9% of the total variance, respectively. The first PC axis discriminated EtOH, 80 and 40 µg L⁻¹ from the other CTZ concentrations. The ordination plots show that the separation of scores along the first axis represents a combination of increased importance of *Infundibura sp.* and decreased importance of *Flagellospora curvula*, *Dimorphospora follicola*, and – to a lesser extent – of other less abundant taxa (*Articulospora tetracladia*, *Lemoniera aquatica*, *Alatospora acuminata*, *Heliscus submersus* and *Dimorphospora follicola*) along the clotrimazole gradient. As the concentration of CTZ increases, the importance of *Infundibura sp.* increases as the other taxa become less abundant (as seen in Figure 19). However, at 80 µg L⁻¹ the importance of *Dimorphospora follicola* increased and *Infundibura sp.* became less abundant (as seen in Figure 19). Although there was some variation along the second axis (e.g., EtOH has lower scores than the other treatments), this axis explains much less of the variance than the first axis.

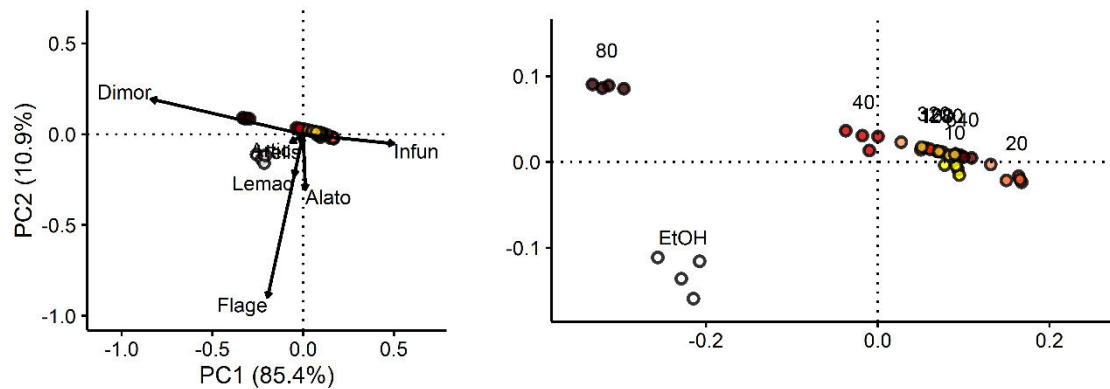


Figure 20 - Ordination plots (PCA on Hellinger-transformed data) of sporulation profiles of aquatic fungi under increasing clotrimazole concentrations, plus a negative control (EtOH). Left panel represents biplot of sample (i.e., experimental units) scores (circles) and species (arrows); right panel represents a zoom of the sample scores, identified by corresponding label near the average score for each experimental treatment. Circles are coloured using a light-to-dark gradient that corresponds to the concentration gradient of each toxicant; EtOH is represented by white circles. Species abbreviations: Flage – *Flagellospora curvula*, Infun – *Infundibura* sp., Artic – *Articulospora tetracladia*, Dimor – *Dimorphospora follicola*, Lemac – *Lemoniera aquatica*, Alato – *Alatospora acuminata* and Helis – *Heliscus submersus*.

3.2.2 Indirect effects of clotrimazole on generalist and specialised invertebrates

Due to the lack of data concerning both environmental detection and ecosystem effects of CTZ, concentrations were chosen according to the previous invertebrate experiment with TBZ, also an azole fungicide. Therefore, $20 \mu\text{g L}^{-1}$ and the $160 \mu\text{g L}^{-1}$ were selected to assess the non-microbial leaf consumption rate, in three different combinations of detritivore invertebrates (Figure 21). After 5 days, the leaf consumption rate in the EtOH control was 0.614, 0.766 and 0.861 g of leaf per g of animal per day for the C, T and C+T treatments, respectively.

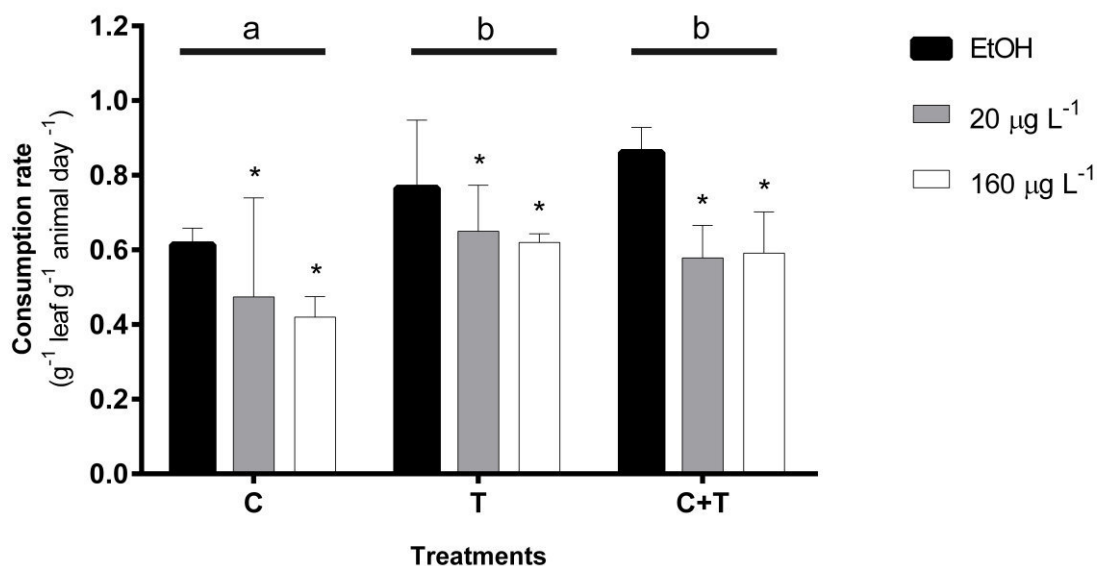


Figure 21 - Consumption rate of alder leaves (previously conditioned with microbiota and exposed to clotrimazole) by *Chironomus riparius* (C), *Allogamus* sp. (T) and both (C+T), after 5 days of exposure in microcosms. Bar colour represents different clotrimazole levels, including a negative control without addition of clotrimazole, but with added carrier (EtOH). Data are shown as mean \pm SD, $n=4$. Different letters (a, b) represent overall differences across invertebrate treatments (C, T, C+T). Asterisks (*) stand for significant differences relatively to EtOH (Dunnett's test, $P < 0.05$).

Leaf consumption rate was significantly affected by the combination of invertebrate species (two-way ANOVA: $MS_{\text{treatment}} = 0.12$, $F_{2, 27} = 7.4$, $P = 0.0027$), as well as by the fungicide concentration (two-way ANOVA: $MS_{\text{concentration}} = 0.15$, $F_{2, 27} = 8.97$, $P = 0.0010$), irrespective of the treatment level (no significant interaction, $MS_{\text{interaction}} = 0.009$, $F_{4, 27} = 0.52$, $P = 0.715$). As shown by the results, exposure to both CTZ concentrations decreased the consumption rate (Dunnett test, $P \leq 0.05$; Figure 21) and leaf consumption was higher in treatments with *Allogamus* sp. (Tukey test, $P \leq 0.05$, Figure 21). Suitable evaluation of the effect of community composition in the C+T treatment was not possible, given that *Allogamus* sp. preyed on *C. riparius* at the initial stage of the experiment.

3.3. Terbinafine

3.3.1 Direct effects of terbinafine on the activity of microbial decomposers

Significant changes were caused by terbinafine in the decomposition of alder leaves inoculated with stream fungi, sporulation rates of aquatic hyphomycetes and microbial community indices (diversity and evenness) – see Table 7. However, post-hoc analysis showed that, in decomposition rates (Figure 22A) and percentage of leaf mass loss (Figure 22B) differences were only found between the CTL and EtOH control, demonstrating the absence of effects of terbinafine in these parameters (Dunnett test, $P \geq 0.05$). Fungal biomass was not significantly affected by the presence of TRF (Figure 22C; Table 7). Nevertheless, terbinafine significantly affected the other parameters, when using the EtOH treatment as a reference (see below).

Sporulation rates of aquatic hyphomycetes was 3.69×10^5 spores per g of leaf mass per day in the CTL, and attained 7.93×10^5 spores per g of leaf mass per day in EtOH control (Figure 22D). TRF exposure significantly affected fungal sporulation (Dunnett test, $P \leq 0.05$), and at 10, 40 and $80 \mu\text{g L}^{-1}$ sporulation rate was significantly higher than that of EtOH, reaching 13.7×10^5 , 11.8×10^5 and 1.23×10^5 spores per g of leaf mass per day, respectively.

Table 7 - One-way ANOVAs on the effects of terbinafine on decomposition rates, leaf mass loss, fungal biomass, sporulation rate and Shannon and Pielou indices.

Parameter	Effect	SS	DF	MS	F	P
Decomposition rates	Concentrations	0,002	9	0,0002	9,55	< 0,0001
	Residuals	0,0007	30	0,00002		
Leaf mass loss	Concentrations	0,029	9	0,003	7,7	< 0,0001
	Residuals	0,0123	30	0,0004		
Fungal biomass	Concentrations	58186	9	6465	1,86	0,1221
	Residuals	66055	19	3477		
Sporulation rate	Concentrations	$3,12 \times 10^{12}$	9	$3,46 \times 10^{11}$	19,91	< 0,0001
	Residuals	$5,22 \times 10^{11}$	30	$1,74 \times 10^{10}$		
Shannon diversity index	Concentrations	3,339	9	0,371	1336	< 0,0001
	Residuals	0,008	30	0,0003		
Pielou evenness index	Concentrations	0,574	9	0,064	57,15	< 0,0001
	Residuals	0,033	30	0,001		

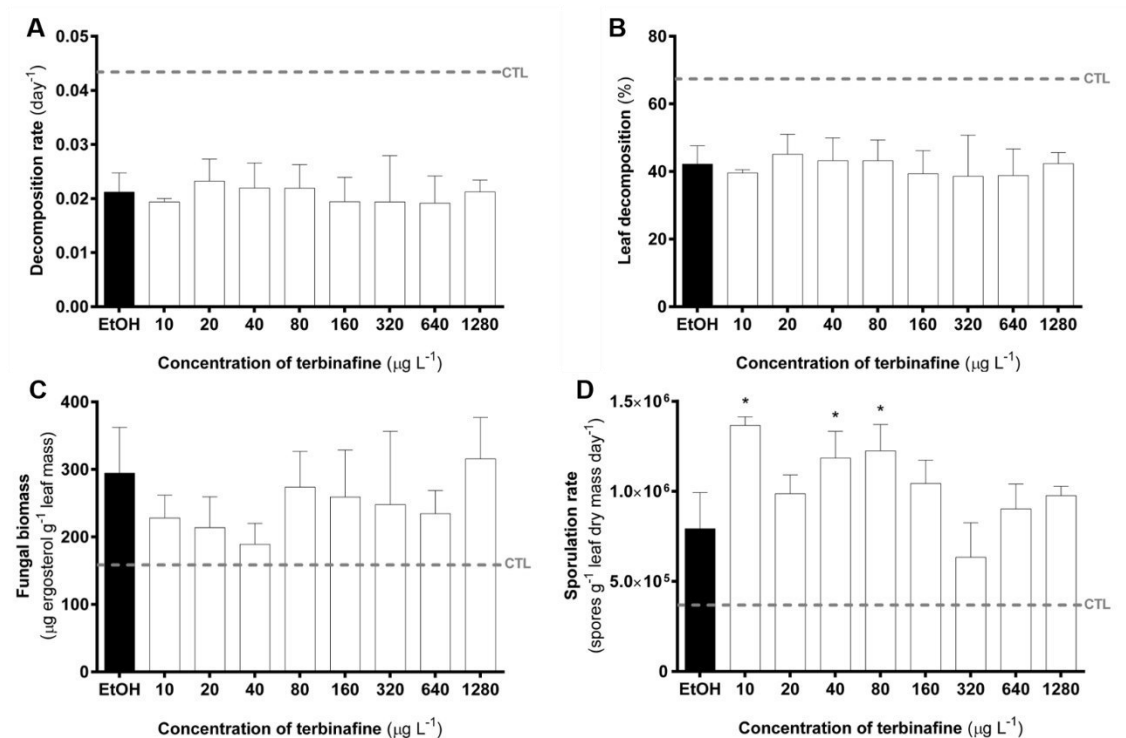


Figure 22 - Effect of terbinafine on (A) decomposition rates of alder leaves, (B) percentage of leaf decomposition, (C) fungal biomass and (D) fungal sporulation after 26 days of exposure in microcosm. Asterisks (*) represent treatments that differ significantly from EtOH control (one-way ANOVA followed by Dunnett test, $P \leq 0.05$). Data are shown as mean \pm SD, $n=4$. Dashed grey line represents the negative control (CTL).

The diversity and evenness of the aquatic hyphomycete community were significantly affected by the presence of TBZ (Dunnett test, $P \leq 0.05$; Figure 23). Shannon's index scored 1.65 and 0.90 in the CTL and EtOH controls, respectively, and decreased under terbinafine stress, regardless of the concentration (Figure 23A). Pielou's evenness controls CTL and EtOH scored 0.83 and 0.49, respectively (Figure 23B). Increasing concentrations of TRF led to an increase of Pielou's evenness scores and results showed that higher values were found at higher concentrations (640 $\mu\text{g L}^{-1}$ - 0.66 and 1280 $\mu\text{g L}^{-1}$ - 0.67).

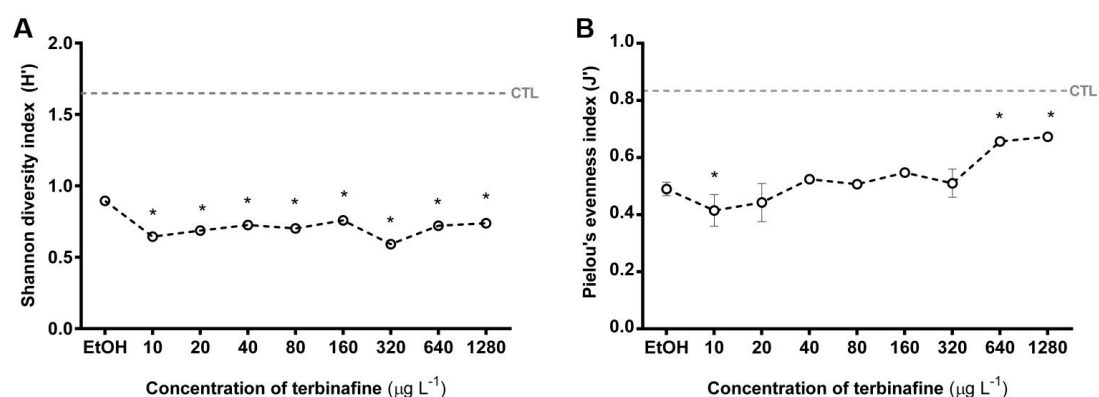


Figure 23 - Effect of terbinafine on (A) Shannon's diversity index (H') and (B) Pielou's evenness index (J') of aquatic hyphomycete community on decomposing alder leaves (based on spore counts), after 26 days of exposure to increasing concentrations of terbinafine in microcosm. Asterisks (*) represent treatments that differ significantly from EtOH control (one-way ANOVA, Dunnett test, $P \leq 0.05$). Data are shown as mean \pm SD, $n=4$. Dashed grey line represents the negative control (CTL).

A total of 8 aquatic hyphomycete species were identified, based on conidial morphology, on decomposing leaves after 26 days in CTL microcosms (Figure 24). The exposure to ethanol decreased species richness to 6 taxa and caused shifts in the species contributions to conidial production. In CTL control, *Flagellospora curvula* (29%), *Articulospora tetracladia* (20 %) and *Infundibura* sp. (24%) were the dominant species, whereas in the EtOH control *Infundibura* sp. (61%) and *Dimorphospora follicola* (31%) were the dominant taxa. In the presence of TRF, the community structure was changed, as the conidial production of *Dimorphospora follicola* increased (45-75%) and that of *Infundibura* sp. decreased (53-22%). At higher concentrations of TRF, each species (*Dimorphospora follicola* and *Infundibura* sp.) represented 50% of the total conidium production. These findings explain the pattern observed for evenness and diversity (see above).

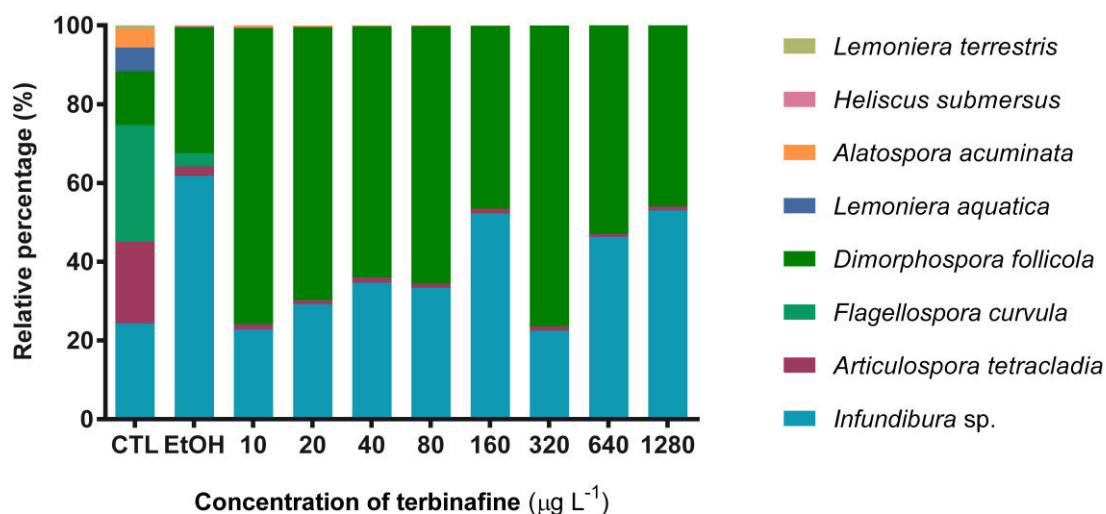


Figure 24 - Effect of terbinafine on the relative contribution of each species to the total conidial production after 26 days of exposure in microcosms. Relative percentage was calculated as the pool of four replicates.

Principal component analysis (PCA) ordination of the eight concentrations and the EtOH, according to sporulation profiles of aquatic fungi, showed that axes 1 and 2 explained 85.7% and 11.4% of the total variance, respectively (Figure 25). The first PC axis discriminated EtOH control, the highest TRF concentrations (160, 640 and 1280 µg L⁻¹) and the lower TRF concentrations. The ordination plots show that the separation of scores along the first axis represents a combination of increased importance of *Dimorphospora follicola* and decreased importance of *Flagellospora curvula*, *Infundibura sp.*, and – to a lesser extent – of other less abundant taxa (*Articulospora tetracladia*, *Lemoniera aquatica*, *Alatospora acuminata* and *Heliscus submersus*) along the terbinafine gradient. As the concentration of TRF increases, the importance of *Dimorphospora follicola* increases as the other taxa become less abundant (as seen in Figure 24). However, as concentration rises (above 160 µg L⁻¹), both *Infundibura sp.* and *Dimorphospora follicola* have similar contributions for the community structure (as seen in Figure 24).

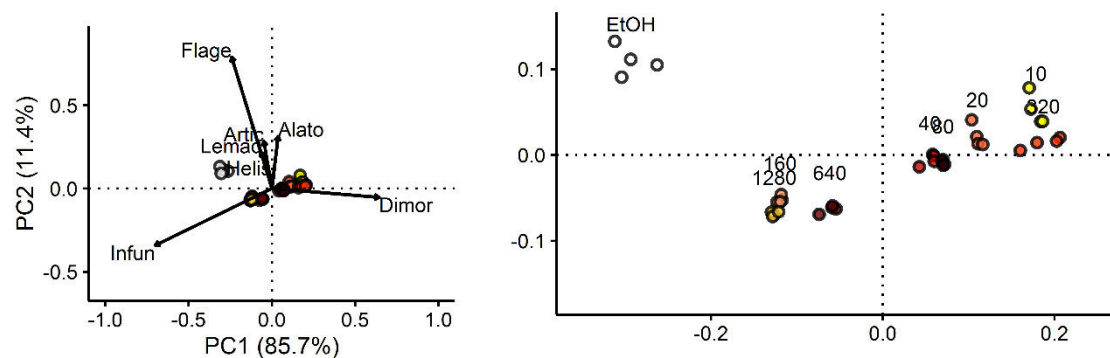


Figure 25 - Ordination plots (PCA on Hellinger-transformed data) of sporulation profiles of aquatic fungi under increasing terbinafine concentrations, plus a negative control (EtOH). Left panel represents biplot of sample (i.e., experimental units) scores (circles) and species (arrows); right panel represents a zoom of the sample scores, identified by corresponding label near the average score for each experimental treatment. Circles are coloured using a light-to-dark gradient that corresponds to the concentration gradient of each toxicant; EtOH is represented by white circles. Species abbreviations: Flage – *Flagellospora curvula*, Infun – *Infundibura* sp., Artic – *Articulospora tetracladia*, Dimor – *Dimorphospora follicola*, Lemac – *Lemoniera aquatica*, Alato – *Alatospora acuminata* and Helis – *Heliscus submersus*.

3.3.2 Indirect effects of terbinafine on generalist and specialised invertebrates

Since there is a lack of data concerning both environmental detection and ecosystem effects of TRF, concentrations were chosen according to the previous invertebrate experiments with TBZ and CTZ. 20 $\mu\text{g L}^{-1}$ and the 160 $\mu\text{g L}^{-1}$ were selected to assess the non-microbial leaf consumption rate, in three combinations of detritivore invertebrates (Figure 26). After 5 days, the leaf consumption rate in the EtOH control was 0.614, 0.766 and 0.861 g of leaf per g of animal per day for the C, T and C+T treatments, respectively.

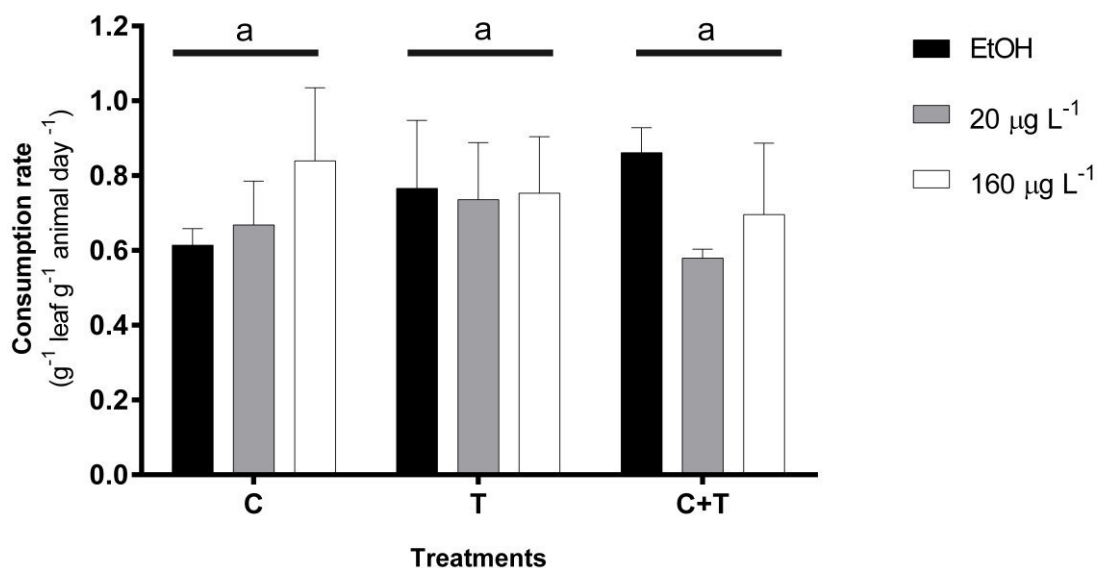


Figure 26 - Consumption rate of alder leaves (previously conditioned with microbiota and exposed to terbinafine) by *Chironomus riparius* (C), *Allogamus* sp. (T) and both (C+T), after 5 days of exposure in microcosms. Bar colour represents different terbinafine levels, including a negative control without addition of terbinafine, but with added carrier (EtOH). Data are shown as mean \pm SD, $n=4$. Different letters (a, b) represent overall differences across treatment means (C, T, C+T). Asterisks (*) stand for significant differences relatively to EtOH (Dunnett's test, $P < 0.05$).

Results showed that consumption rate was not affected by neither the combination of invertebrate species (two-way ANOVA: $MS_{\text{treatment}} = 0.007$, $F_{2, 27} = 0.37$, $P = 0.6964$) nor TRF concentrations (two-way ANOVA: $MS_{\text{concentration}} = 0.036$, $F_{2, 27} = 1.86$, $P = 0.1744$), irrespective of the treatment level (no significant interaction, $MS_{\text{interaction}} = 0.05$, $F_{4, 27} = 2.60$, $P = 0.059$). Notwithstanding, a suitable evaluation of the effect of community composition in the C+T treatment is not possible, given that *Allogamus* sp. preyed on *C. riparius* at the initial stage of the experiment.

4. General discussion

In low order forested streams, where insufficient sunlight penetrates through the canopies, plant litter from the riparian vegetation constitutes the major source of energy for freshwater organisms (Webster & Benfield 1986). The decomposition of this material is a key process in freshwater ecosystems and its main drivers are aquatic microbial decomposers and invertebrate shredders (Gessner, Chauvet & Dobson 1999). Fungi, particularly aquatic hyphomycetes, seem to play a major role in organic matter turnover and constitute a significant link in detrital food webs between plant-litter and stream invertebrates (Graça 2001; Pascoal, Cássio & Marcotegui 2005). Fungal activity enhances leaf nutritional value and palatability to shredder consumption (Suberkropp 1998b). Some studies have supported that fungal diversity is important for litter decomposition in freshwaters (Bärlocher & Corkum 2003; Duarte *et al.* 2006) and the variability of this process is lower when diversity is higher (Dang, Chauvet & Gessner 2005; Pascoal *et al.* 2010). However, anthropogenic stress might change diversity, composition and activity of aquatic hyphomycete communities and consequently affect detrital-based food webs. Human activities and population growth are threatening biodiversity and altering ecosystems at a worldwide scale (Vitousek *et al.* 1997). In particular, the widespread and extensive use of antifungal formulations, which include agrochemicals and pharmaceuticals, may affect microbial decomposer communities. The input of fungicides into freshwaters, from various sources (agrochemicals, pharmaceuticals, industrial additives), is exposing freshwater ecosystem to enormous pressure. We focused our study on the impacts of fungicides on freshwater biota because these chemicals pose a major threat to the aquatic microbial community that can upscale to higher trophic levels, affecting ecosystem processes (Montuelle *et al.* 2010; Rasmussen *et al.* 2012).

We investigated the effects of tebuconazole (agrochemical), clotrimazole and terbinafine (pharmaceuticals) on freshwater microbial communities, invertebrates and leaf-litter decomposition.

4.1. Effects of carrier (ethanol) on microbial communities and leaf decomposition

Ethanol was used as a carrier for all fungicides, as their limit of solubility in water was low. Whereas the fungicide concentrations used in our study were within the solubility limit of the tested substances, the need for preparing stock solutions (i.e., more concentrated) required the use of this solvent. Our study shows that ethanol

decreased leaf decomposition, and stimulated fungal biomass and sporulation. Identification of spores released from decomposing leaves showed that ethanol altered the composition of the aquatic hyphomycete community, and decreased overall richness and diversity when compared to the CTL (with no ethanol and no fungicide). For instance, *Infundibura* sp. was stimulated by ethanol, replacing *A. tetracladia* and *F. curvula* as the dominant species. Other studies conducted with this carrier present some controversial results. Moreirinha and colleagues (2011) found no effects of ethanol at 0.3% v/v on leaf mass loss, fungal biomass, and sporulation. However, at 0.1% v/v, Barros and colleagues (2016) found that leaf decomposition and fungal sporulation were inhibited and fungal biomass was stimulated. Our data confirm the findings of Barros et al. (2016). Ethanol can potentially be used as an easily-available carbon and energy source by microbes, explaining the decrease in leaf consumption by fungi and the increased fungal biomass. Moreover, ethanol has the ability to affect the morphology of fungi (Canetta, Adya & Walker 2006), and the activity of extracellular enzymes produced by aquatic hyphomycetes – responsible for the degradation of structural polymers of leaf detritus (Suberkropp 1998b), which might be inhibited by ethanol (Chen & Jin 2006). Given the evidence that ethanol affected all the parameters we measured, all results of fungicide exposure were compared to EtOH to distinguish the isolated effects of toxicants (i.e., removing the influence of the carrier).

The discrepancy between the various works studying the effect of ethanol may be explained by differences in composition of fungal communities across studies or differences in species richness of communities that presented different sensitivity to ethanol (Barros *et al.* 2016). This large variability in community responses is the downside of working with natural communities (leaves are colonized by local microbia and subject to all sorts of random variation, including spatial, seasonal, etc.). However, there is an enormous advantage of using this approach, as it is more realistic and allows measuring community responses and ecosystem processes (Bärlocher 1992).

4.2. Direct effects of fungicides on microbial communities and leaf decomposition

Our results show that all tested fungicides can have impacts on microbial communities. Exposure to tebuconazole and clotrimazole strongly reduced fungal biomass (up to 57% and 68%, respectively) and sporulation rates (up to 92% and 77%, respectively). Terbinafine, on the other hand, stimulated fungal sporulation (up to 70%) and caused no measurable alterations on fungal biomass. This partially agrees with

earlier reports showing that, in the presence of tebuconazole, fungal sporulation and biomass associated with decomposing leaves decrease (Bundschuh *et al.* 2011; Zubrod *et al.* 2011). The effects of clotrimazole (imidazole antifungal) on the aquatic microbial community are yet to be investigated, however, results similar to that of tebuconazole (triazole) were expected, given their similar mode of action. Azole fungicides are considered fungistatic agents (Hof 2001), since they act by preventing the demethylation of lanosterol in ergosterol, disrupting fungal growth. Moreover, studies with other imidazole fungicides also suggest that fungal biomass and sporulation can be affected by these fungicides (Flores *et al.* 2014). Concerning terbinafine, this is one of the first studies that assessed its effects on aquatic hyphomycetes; as such, no data is available for comparison. As terbinafine acts on early steps of ergosterol biosynthesis, inhibiting the squalene epoxidase responsible for the catalysis of squalene epoxidation, effects on the microbial community were expected to be more accentuated mainly because the primary action of allylamines is fungicidal, resulting of the toxic accumulation of squalene (Ryder, 1992). However, our results showed that terbinafine was not as harmful as the azole compounds and stimulated fungal sporulation. Lack of information regarding the environmental fate of terbinafine in aquatic ecosystems restricts the speculation about possible causes for these findings.

The analysis of aquatic hyphomycete communities, based on identification of released spores from decomposing leaves, also showed shifts in community composition after exposure to tebuconazole, clotrimazole and terbinafine. The number of sporulating taxa decreased, suggesting that some species were depleted with exposure to the fungicides, or their reproduction was inhibited. Exposure to the azole fungicides increased the relative contribution of *Infundibura* sp. to the total conidium production, becoming the dominant species (contributing with more than 90%), whilst most of the others disappeared. Terbinafine also reduced the number of sporulating taxa but it increased species equitability by reducing the dominance of *Infundibura* sp.. The exposure to terbinafine resulted in an impoverishment of the fungal community, but not as marked as in the other fungicides. The assessment of the sporulation of fungal community can give an unrealistic image about the composition of the fungal community colonising the leaves. This parameter only allows us to acquire knowledge about the effects of the fungicides on fungi that are able to reproduce and to evaluate how these chemicals can affect the composition of future communities. A more accurate insight about the effects on the structure of the effective community colonizing the leaves could be achieved by using DNA fingerprinting techniques, such denaturing gradient gel electrophoresis (DGGE).

Even though loss of fungal species has been associated with some anthropogenic disturbances, this was not always accompanied by changes in leaf decomposition (e.g. Lecerf & Chauvet 2008). We found that the presence of all fungicides had no effects on leaf decomposition rates. Most studies indicate that fungal diversity and reproduction are more affected by fungicides than fungal biomass or leaf decomposition (Bundschuh *et al.* 2011; Zubrod *et al.* 2011). The response to pollution is not standard, and other pollutants such as metals are widely reported to inhibit leaf decomposition (e.g. Sridhar *et al.* 2001), fungal diversity and reproduction (e.g. Sridhar *et al.* 2001; Baudoin *et al.* 2008), whilst not affecting fungal biomass (Duarte, Pascoal & Cássio 2004; Baudoin *et al.* 2008). It seems that decomposition and fungal biomass are overall less responsive to fungicides, unlike sporulation. The impoverishment of the fungal community and the reduced sporulation rates (for the azole fungicides) was not accompanied by decreased leaf decomposition, suggesting that the contribution of some species might compensate the depletion of others. Indeed, functional redundancy among fungal species is an important way to maintain ecological functions in streams under stress (Pascoal, Cássio, & Marvanová, 2005). The ecological roles of different species overlap and this compensation masks the function degradation, creating a non-linear relationship between species richness and ecosystem function. The redundancy hypothesis foresees a positive asymptotic relationship, provided that the loss of some species is counterbalanced by others within the same functional group (Walker 1992). Evidence of species redundancy has been reported to terrestrial (Laakso & Setälä 1999; Cragg & Bardgett 2001) and aquatic ecosystems (Bärlocher 1998; Pascoal, Cássio & Marvanová 2005).

4.3. Indirect effects of fungicides on leaf consumption by invertebrates

Feeding behaviour of invertebrates is one of the most sensitive monitoring tools in ecotoxicology for assessing sub-lethal effects of contaminants (Pestana *et al.* 2009). The combination of the decomposer-shredder-collector model with leaf litter decomposition can contribute towards a better understanding of the indirect effects of fungicides on species interactions and ecosystem functioning. The use of species with different degrees of specialization can help to further understand if niche overlap can compensate the effects of contamination; however, as *Allogamus* sp. preyed on *Chironomus riparius* (in the C+T treatment), evaluation of the effect of different degrees of specialization was not fully possible to assess. Aggressiveness of Trichoptera larvae has been reported in several studies. Wissinger and collaborators (2004) found that

aggression and cannibalism is greater in species that live in habitats that are non-permanent (which is not our case). This shift in the feeding habits can be an adaptation to environmental change. In the lab, when both species were forced to coexist in an unnatural and small space, we observed a response similar to that found by Wissinger and collaborators (2004) was observed.

Feeding behaviour of *C. riparius* and *Allogamus* sp. was not affected in the presence of tebuconazole- and terbinafine-contaminated leaves. Considering that fungal biomass was inhibited by tebuconazole, differences were expected in leaf consumption by invertebrates. The palatability of leaf material is very important to stream invertebrates, including Trichoptera, and alterations in leaf consumption were described when diversity and biomass of microbial community change (Arsuffi & Suberkropp 1989; Graça 2001). Hence, we can speculate that the decrease in fungal biomass was not accentuated enough to decrease palatability. In a study by Bundschuh and colleagues (2011) the exposure to tebuconazole decreased the consumption rate of Gammarids. However, the differences between the species (*Gammarus fossarum* vs *Allogamus* sp. and *C. riparius*) and the higher concentrations used by Bundschuh and colleagues (2011) (up to 500 $\mu\text{g L}^{-1}$) can explain the discrepancies found with our study.

The feeding behaviour of *C. riparius* and *Allogamus* sp. decreased in the presence of clotrimazole contaminated leaves. Considering the results of the initial experiment (with microbes only) and the results of the feeding behaviour of the invertebrates exposed to the other fungicides, it is difficult to explain the results to clotrimazole exposure. Fungal biomass associated with the leaves exposed to clotrimazole decreased; however, the decrease was comparable to that resulting from tebuconazole exposure. Since no differences were found in the invertebrate feeding activity on tebuconazole-contaminated leaves (as well as terbinafine-contaminated leaves), the clotrimazole results may be a statistical artifact or a consequence of differences in the microbial community colonizing the leaves. The assessment of microbial community composition can help to clarify the differences between the effects of fungicides. Although we observed effects on fungal reproduction, we cannot distinctly attribute them to shifts in the extant microbial community colonizing the leaves (see 4.2).

In all experiments, the consumption rate of *Allogamus* sp. (0.766 g of leaf per g of animal per day) was within the typical range reported for stream invertebrate shredders (0.04 to 0.5 mg of leaf per mg of animal per day; Arsuffi & Suberkropp, 1989). The feeding activity of *C. riparius* reinforced the idea that they are not exclusive collectors, as they consumed leaves, yet at a lower rate than *Allogamus* sp. (0.614 g of

leaf per g of animal per day). Differences in consumption rates between the two invertebrate species were detected in the tebuconazole and clotrimazole experiment but not in the terbinafine experiment. However, when only the EtOH control was analysed in the terbinafine experiment, the tendency does seem for a lower consumption rate with *C. riparius* (i.e., in the absence of *Allogamus* sp.).

4.4. Management implications

Continuous and extensive application of fungicides can severely impact water quality, and biota that can be exposed during large periods of time (Berenzen *et al.* 2005; Bereswill *et al.* 2012). The intensification of agricultural exploitations over the last century, as a response for human population growth and needs, exposes ecosystems to enormous pressure. In the last year, 8244.4 tonnes of fungicides were sold in Portugal, representing 64% of the total pesticide sales (Eurostat, 2016; INE, 2015). Spillage from agricultural activities may impair the surrounding terrestrial and aquatic systems (Stoate *et al.* 2009), and the impacts of agricultural fungicides on aquatic biota has been widely explored (e.g. Zubrod *et al.* 2011, 2014; Flores *et al.* 2014). However, agriculture is not the only environmental source of fungicides. Sales of antifungal pharmaceuticals in Portugal reached 8.981.280 € in 2013 (INFARMED, 2015). However, data on the occurrence of these antifungal agents in the environment is limited and the impacts on freshwater biota are still poorly explored, especially in what concerns non-target fungi that play important ecological functions and are preferential targets of these substances (Zubrod *et al.* 2015). The incomplete degradation of pharmaceuticals during the wastewater treatment causes contamination in effluents (Hirsch *et al.* 1999) and the application of sewage sludge can contaminate the lands and water bodies if runoff occurs (Boxall 2004). Pharmaceuticals are detected in natural ecosystems at low concentrations (e.g. Kahle *et al.* 2008), but since these antifungals are created to be very effective against fungal infections, the effects on aquatic biota can be high. Hence, the monitoring of antifungal pharmaceuticals in streams and their effects on freshwater biota is of extreme importance, and should walk hand-by-hand with the monitoring of its agrochemical “siblings”.

Overall, our results indicate that all fungicides can have toxic effects on aquatic hyphomycete communities. Tebuconazole and terbinafine were the most and least harmful compounds for the aquatic hyphomycete community, respectively. Many times, the effects on the microbial community are overlooked. Nevertheless, the fungicide-

induced alterations on the sporulating aquatic fungi can bear long-term consequences to ecosystem functions. The modification of future fungal communities can alter leaf conditioning and consequently the feeding activity of higher trophic levels. As the feeding activity of invertebrates on the detritus is highly dependent on the leaf nutritional value, the depletion of fungal species can constrain the entire food chain and ecosystem processes. Even though no indirect effects on higher trophic levels were detected in our study, it is advisable to consider the possibility of indirect effects of contaminants on non-target species.

Regulatory risk assessment of toxic chemicals on biota relies on ecotoxicological studies to predict the negative impacts of such stressors. Standard ecotoxicology tests are used as tools to predict the direct effects of contaminants on natural populations in order to estimate permissive levels of contamination and generate data for environmental regulation (Long *et al.* 1995; Rohr, Kerby & Sih 2006). The generated data is extremely important for regulatory authorities and legislators, promoting a more informed choice of anti-fungal compounds to consumers (pharmaceuticals and agrochemicals). Detailed information about the selectivity of antifungals (for the pest fungus) and possible disruptive effects to aquatic ecosystems (including aquatic hyphomycetes), on which we strongly depend, can help to mitigate the enormous anthropogenic pressure to which freshwater ecosystems are exposed.

5. References

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